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12. PERSONAL AUTHOR(S) GILLETTE, Martha; CHEN, Dong; DEMARCO, Steven; DING, Jian; FAIMAN, Lia; GALLMAN, Eve; MEDANIC, Marija; MICHEL, Ann-Marie; REA, Michael (USAF/SAM); RICHARD, Dan; TCHENG

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
Our research addresses the cellular organization and regulation of a biological clock that controls daily (circadian) rhythms of behavior (e.g., performance), physiology and metabolism in mammals. This clock, located in the brain's suprachiasmatic nucleus (SCN), can be removed in a slice of hypothalamus, maintained in a life support system for up to 3 days and studied directly. Using this approach, progress in the 3 1/2 years of this award has been made in: 1) localizing time-keeping properties within the SCN of rat, 2) identifying electrophysiological properties of neurons in the major SCN subdivisions, 3) establishing regulatory roles for serotonin, a neuromodulatory input from the brain's arousal center in the raphe nuclei, as well as for neuropeptide Y, an input from the intergeniculate area, 4) determining the level of glutamic acid decarboxylase (GAD), the biosynthetic enzyme for the inhibitory neurotransmitter GABA, in SCN over the circadian cycle, and 5) examining the involvement of glutamate/nitric oxide signal transduction in mediating light entrainment of the SCN through retinal inputs. This project involves both individual and interactive research projects at the University of Illinois and the USAF School of Aerospace Medicine.

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THE ORGANIZATION OF THE SUPRACHIASMATIC CIRCADIAN PACEMAKER OF THE RAT AND ITS REGULATION BY NEUROTRANSMITTERS AND MODULATORS

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THE ORGANIZATION OF THE SUPRACHIASMATIC CIRCADIAN PACEMAKER OF THE RAT AND ITS REGULATION BY NEUROTRANSMITTERS AND MODULATORS

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RESEARCH OBJECTIVES

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is a circadian pacemaker that serves a well-defined, critical role in the generation and entrainment of daily rhythms of physiological, metabolic and behavioral functions. The SCN contain an endogenous pacemaker that generates near 24-hr SCN rhythms of electrical activity and vasopressin secretion. Outputs from this central pacemaker time cellular, tissue and organismic circadian rhythms. All circadian rhythms are reset by changes in environmental lighting, which can affect the SCN through inputs from the retina, intergeniculate leaflet or the raphe. However, little is known about the way in which the neuronal components of the SCN are organized to carry out time-keeping or to analyze phase-resetting information. This study sought to determine 1) the functional organization of the SCN by electrophysiological analyses of regional distribution of pacemaking properties and neuronal characteristics, as well as, 2) SCN responses to extrinsic and intrinsic neurotransmitters and modulators.

We used the rat hypothalamic brain slice to study the functional organization of the SCN directly. Our work has established that circadian pacemaking and resetting properties are endogenous to the SCN and can be studied *in vitro*. In the studies undertaken during this award, the circadian rhythm of SCN electrical activity was recorded continuously in intact and microdissected slices of rat hypothalamus for 32 hr after slice preparation. Persistence of a rhythm in microdissected subregions was determined. Whole cell recording in slice of single SCN neurons was performed over the circadian cycle to assess the range of electrophysiological characteristics in each SCN region together with diurnal changes in electrical properties. The neuromodulators serotonin and neuropeptide Y were applied focally with micropipette, and effects on the phasing of the electrical activity rhythm determined for 24-48 hr after treatment. Additionally, the levels of glutamic acid decarboxylase (GAD), the biosynthetic enzyme for the inhibitory neurotransmitter GABA, was assessed over the circadian cycle. In experiments directed at understanding regulation by retinohypothalamic afferents, Dr. Rea's lab at the USAF-SAM has examined release of excitatory amino acids, field potential activity and pharmacological blockade in SCN upon stimulation of the optic nerve. and nitric oxide synthase (NOS), a possible mediator of glutamate stimulation. Finally, we have collaborated with Dr. Rea in experiments that identify components of the mechanism by which light, the major environmental stimulus for entrainment of daily rhythms, adjusts the SCN. Our studies together demonstrate that glutamate, working through nitric oxide-a cyclic AMP binding protein phosphorylation-c*fos* induction, in the major regulator of circadian rhythms at night.

The main hypotheses tested in this study were: 1) pacemaking properties are distributed throughout the SCN; 2) the neurons of the SCN are homogeneous with respect to their electrical and pacemaking properties; 3) neuromodulators from inputs implicated in phase-shifts of behavior by dark pulses (serotonin from the raphe, neuropeptide Y from the intergeniculate) are effective phase-shifting agents for SCN during the circadian day; 4) GAD levels are constant over the circadian cycle; and 5) light information carried by the retinohypothalamic tract affects the SCN via excitatory amino acids (*viz.*, glutamate).

The long-term goal of these studies has been to understand how neurons of the SCN are organized to generate a 24-hr biological clock and what role specific neurotransmitters and modulators play in the pacemaking and resetting process. Because the SCN integrate most circadian behaviors and metabolic fluxes, this study has basic relevance to understanding circadian dysfunction induced by transmeridian travel and sustained, irregular work

schedules, with application to improving human performance under conditions that induce circadian desynchronization.

In accord with a directive from Dr. Haddad, Program Manager of the Chronobiology Initiative, the body of this report will be our publication in *Journal of Biological Rhythms* vol. 8, Supplement, 1993, pp. S53-S58, which follows.

RESEARCH ARTICLES PUBLISHED OR PLANNED FOR TECHNICAL JOURNALS

Medanic, M. and **M.U. Gillette.** 1992. Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker *in vitro* during the daytime. **J of Physiology (London)** 450: 629-642.

Gillette, M.U., S.J. DeMarco, J.M. Ding, E.A. Gallman, L.E. Faiman, C. Liu, A.J. McArthur, M. Medanic, D. Richard, T.K. Tcheng and E.T. Weber. 1993. The organization of the suprachiasmatic circadian pacemaker of the rat and its regulation by neurotransmitters and modulators. **J of Biological Rhythms**, 8: S53-S58.

Medanic, M. and **M.U. Gillette.** 1993. Suprachiasmatic circadian pacemaker of rat shows two windows of sensitivity to neuropeptide Y *in vitro.* **Brain Research,** 620: 281-286.

Gallman, E.A. and **M.U. Gillette.** 1993. Diversity and circadian modulation of suprachiasmatic neurons studied by whole cell recording in rat brain slice. In revision.

Satinoff, E., H. Li , T. Tcheng , C. Liu, A. McArthur, M. Medanic and **M.U. Gillette.** 1993. Do the SCN oscillate in old rats as they do in young ones? **American Journal of Physiology,** 265: R1216-R1222..

Gillette, M.U., M. Medanic, A.J.McArthur, C. Liu, J.M. Ding, T.K. Tcheng, and E.A. Gallman. 1994. Electrophysiology of the SCN Clock: Intrinsic Neuronal Rhythms and their Adjustment. Ciba Foundation Symp. 183. **Circadian Clocks and their Adjustment**. J. Waterhouse, Org. , Wiley, Chichester, In press

DeMarco, S.J., D. Richard, L. Faiman and **M.U. Gillette.** 1994. Circadian regulation of g-amino decarboxylase levels and activity in the rat suprachiasmatic nucleus *in vitro.* **J of Neurochem** Prepared for submission.

Ding, J.M., Chen, D, Faiman, L.E. and **M.U. Gillette.** 1994. Light-like regulation of the suprachiasmatic circadian clock by a glutamate-nitric oxide pathway to CREB. Prepared for submission.

PARTICIPATING PROFESSIONALS

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Marija Medanic, Systems and Integrative Training Grant Trainee and Graduate Research Assistant, Department of Physiology & Biophysics, University of Illinois; M.S. awarded in Jan., 1991; Pursuing Ph.D. currently.

Ann-Marie Michel, Research Specialist in Biological Science; working at USAF School of Aerospace Medicine; appointed through the University of Illinois

Daniel Richard, Undergraduate Distinction Student, Department of Cell & Structural Biology, University of Illinois

Thomas K. Tcheng, Graduate Research Assistant, Neuroscience Program, University of Illinois; currently taking Qualifying Examination for the Ph.D. program.

Todd Weber, Air Force Graduate Fellow, pursuing Ph.D. in Physiology & Biophysics, University of Illinois.

INTERACTIONS THROUGH MEETINGS AND COLLABORATIVE EXPERIMENTS

MEETINGS

Gillette, M.U. and Tcheng, T.K. 1990. Localization of a circadian pacemaker tothe ventrolateral suprachiasmatic nucleus (SCN). Presented at the Society for Research on BIological Rhythms, May, 1990. Amelia Island, FL.

Tcheng, T.K. and Gillette, M.U. 1990. Electrical characterization of ventrolateral and dorsomedial regions of the suprachiasmatic nucleus. Presented at eh Society for Neuroscience Meeting, October, 1990, St. Louis, MO.

Medanic, M. and Gillette, M.U. 1990. Serotonin phase shifts the circadian rhythm of electircal activity in the rat SCN *in vitro*. Presented at the Society for Neuroscience Meeting, October, 1990, St. Louis, MO.

Gillette, M.U. 1991. Cellular regulators of the SCN pacemaker studied in the brain slice. Presented as part of a panel, "Current Status of Circadian Rhythm Regulation in Mammals", at the 1991 Winter Conference for Brain Research at Vail, CO. Other members of the panel included L. Morin (SUNY-Stony Brook), D. Earnest (Rochester) and M. Lehman (Cinncinati).

Gillette, M.U. 1991. Analysis of phase-locked regulators of circadian rhythms in the SCN brain slice. Invited lecture presented to the Gordon Conference on Chronobiology, Irsee, Germany, October, 1991.

Tcheng, T.K. and Gillette, M.U. 1991. Characterization of regional neuronal activity in the suprachiasmatic nucleus using a curve-fitting technique. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Gallman, E.A., Nolan, P.C., Waldrop, T.G. and Gillette, M.U. 1991. Whole cell recording of neurons of the suprachiasmatic nucleus (SCN) studied in rat brain slice. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Medanic, M. and Gillette, M.U. 1991. Serotonin agonists advance the circadian rhythm of neuronal activity in rat SCN *in vitro*. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Richard, D., Faiman, L. and Gillette, M.U. 1991. Quantitation of glutamic acid decarboxymase (GAD) and tyrosine hydroxylase (TH) in single suprachiasmatic nuclei (SCN) across the circadian cycle. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Gillette, M.U. 1991. Aminergic and amino acid transmitters in the control of SCN rhythms. Invited lecture presented at the Conference Philippe Laudat "Neurobiology of circadian and seasonal rhythms: animal and clinical studies", November, 1991, Strasbourg, France

Gillette, M.U., McArthur, A.J., Liu, C. and Medanic, M. 1992. Intrinsic organization of the SCN circadian pacemaker studied by long-term electrical recording *in vitro*. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Satinoff, E., Li, H., McArthur, A.J., Medanic, M., Tcheng, T., and Gillette, M.U. 1992. Does the SCN oscillate in old rats as it does in young ones? Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Gallman, E.A. and Gillette, M.U. 1992. Whole cell recording of neurons of the suprachiasmatic nucleus (SCN) studied in rat brain slice. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Medanic, M. and Gillette, M.U. 1992. NPY phase-shifts the circadian rhythm of neuronal activity in the rat SCN *in vitro*. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Gillette, M.U. 1993. Circadian patterns of suprachiasmatic neuronal activity and sensitivity. Invited presentation at the World Federation of Sleep Research Societies meeting on the Cellular Consequences of Sleep, Maui, HI, March, 1993.

Ding, J.M. and Gillette, M.U. 1993. Glutamate induces light-like phase shifts in the rat SCN in brain slice. Presented at the Society for Neuroscience Meeting, Nov., 1993, Washington, D.C.

Gallman, E.A. and Gillette, M.U. 1993. Circadian modulation of membrane properties of SCN neurons in rat brain slice. Presented at the Society for Neuroscience Meeting, Nov., 1993, Washington, D.C.

Medanic, M. and Gillette, M.U. 1993. Rat suprachiasmatic circadian pacemaker shows two windows of sensitivity to NPY *in vitro*. Presented at the Society for Neuroscience Meeting, Nov., 1993, Washington, D.C.

Satinoff, E. Li, H, Liu, C., McArthur, A.J., Medanic, M., Tcheng, T. and Gillette, M.U. 1993. Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? Presented at the Society for Neuroscience Meeting, Nov., 1993, Washington, D.C.

Gillette, M.U., 1993. Electrophysiology of the SCN Clock: Intrinsic Neuronal Rhythms and their Adjustment. Presented at Ciba Foundation Symp. 183. Circadian Clocks and their Adjustment, at Ciba House, London, England, September, 1993.

COLLABORATIONS

With Dr. Mike Rea (USAF-SAM, Brooks AFB) we have undertaken a number of collaborative experiments. The most productive interactions have been oriented around the hypothesis that light induced phase-shifts might be mediated through glutamate stimulation of nitric oxide synthase, followed by activation of guanyl cyclase. These should lead to induction of phosphorylation of cyclic AMP response element binding protein (CREB) and Fos expression in the SCN. Todd Weber, an Air Force Graduate Fellow in my lab, has made a number of attempts to measure cGMP levels stimulated by glutamate in cerebellum, the tissue in which this pathway was first described (Bredt and Snyder, 1991.) These have produced highly variable results; since Todd is extremely experienced in this technique, we have put the project on hold while we try to implicate NO synthase more directly in light-induced phase-shifts. This fall, Todd spent 3 months in Dr. Mike Rea's lab at USAF-SAM where he conducted experiment that strongly implicate nitric oxide in the signal transduction pathway by which light reset behavioral rhythms in hamsters. This work is fully complementary to the studies of Dr. Ding reported above. Todd, Dr. Rea and I are preparing a manuscript reporting these findings presently.

I have valued the intelluctual company of several members of the group funded by AFOSR including Mike Rea, Ed Dudek and David Earnest. They have been ever available for consultation or discussion of methods and results in these studies.

SUMMARY OF PROGRESS

1) The preponderance of data suggest that the SCN pacemaker is relatively distributed and is organized primarily in the VL-SCN, the region receiving afferent fibers from regulatory brain regions.

2) Neurons of the SCN are not homogeneous, but rather represent a diverse population with a range of electrophysiological characteristics. Based on other descriptive studies of pacemaker neurons in other brain regions, some of these characteristics might be expected to be found in oscillatory neurons.

3) Serotonin and NPY are both potent regulators of the SCN when briefly and focally applied. They induce phase-advances during the daytime portion of the circadian cycle only; at nighttime they are without effect when applied focally to the site that raphe afferents terminate. Serotonin appears to act through a $5HT_{1A}$-like receptor via a pertussis-sensitive G protein. Half maximal responses are achieved at 10^{-9} M.

4) Glutamic acid decarboxylase (GAD) levels undergo significant diurnal variation over the circadian cycle. Initial experiments suggest it unlikely that nitric oxide synthase links glutamate receptor stimulation to enhanced cGMP levels.

5) Glutamate applied directly to the SCN produces a light-like phase response curve, and activates a nitric oxide generating pathway that leads to CREB-P. This supports the evidence that the effects of light on circadian rhythms of animals is mediated by glutamate at the retinohypothalamic tract afferents to the SCN.

M.U. GILLETTE AFOSR 90-0205

Journal of Biological Rhythms, Vol. 8, Supplement, 1993, pp. S53–S58

The Organization of the Suprachiasmatic Circadian Pacemaker of the Rat and Its Regulation by Neurotransmitters and Modulators

Martha U. Gillette,[*,†,‡] *Steven J. DeMarco,*[*] *Jian M. Ding,*[*,‡] *Eve A. Gallman,*[*,‡] *Lia E. Faiman,*[*] *Chen Liu,*[‡] *Angela J. McArthur,*[†] *Marija Medanic,*[†] *Daniel Richard,*[*] *Thomas K. Tcheng,*[‡] *and E. Todd Weber*[†]

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Abstract The long-term goal of our research is to understand how cells of the suprachiasmatic nucleus (SCN) are organized to form a 24-hr biological clock, and what roles specific neurotransmitters and modulators play in timekeeping and resetting processes. We have been addressing these questions by assessing the pattern of spontaneous neuronal activity, using extracellular and whole-cell patch recording techniques in long-lived SCN brain slices from rats. We have observed that a robust pacemaker persists in the ventrolateral region of microdissected SCN, and have begun to define the electrophysiological properties of neurons in this region. Furthermore, we are investigating changing sensitivities of the SCN to resetting by exogenous neurotransmitters, such as glutamate, serotonin, and neuropeptide Y, across the circadian cycle. Our findings emphasize the complexity of organization and control of mammalian circadian timing.

Key words brain slice, glutamate, neuropeptide Y, rat, serotonin, suprachiasmatic nucleus, ventrolateral SCN, whole-cell patch recording

The central role of the suprachiasmatic nuclei (SCN) in the mammalian circadian system is well established. An endogenously pacemaking tissue, the SCN exhibit a near-24-hr period in intrinsic rhythms of electrical activity and vasopressin secretion (Earnest and Sladek, 1987; Gillette and Reppert, 1987; Prosser and Gillette, 1989). Outputs from this central pacemaker supply a time base for circadian rhythms in cellular, tissue, and organismic functions. Behavioral circadian rhythms are reset differentially over the 24-hr circadian cycle by variables that include environmental lighting (DeCoursey, 1964; Boulos and Rusak, 1982) and activity state (Mrosovsky and Salmon, 1987). These phase-resetting stimuli must affect the SCN through input pathways, such as those from the retina (Moore and Lenn, 1972), intergeniculate leaflet (Swanson et al., 1974; Card and Moore, 1982), or dorsal raphe (Aghajanian et al., 1969; Moore et al., 1978). However, little is known about the way in which the cellular components of the SCN are organized to carry out timekeeping or to analyze phase-resetting information. We have been seeking to determine (1) the functional organization of the SCN through electrophysiological analyses of regional distribution of pacemaking properties and neuronal characteristics, as well as (2) the circadian nature of SCN pacemaker regulation by neurotransmitters and modulators.

We have been using the hypothalamic brain slice (Hatton et al., 1980) to study the functional organization of the SCN directly. Slices are prepared from Long–Evans rats,

raised to 5–10 weeks of age on a light–dark cycle (LD 12:12) in our inbred colony. Our previous work established that circadian pacemaking and resetting properties are endogenous to the SCN in slices and can be analyzed *in vitro* (for a review, see Gillette, 1991). The circadian rhythm of SCN electrical activity was recorded extracellularly in intact and microdissected slices of rat hypothalamus for 1–3 days after slice preparation. Persistence of a rhythm in microdissected subregions was examined. Whole-cell patch recording in slices (Blanton et al., 1989) of single SCN neurons was performed over the circadian cycle to assess the range of electrophysiological features of SCN neurons, together with diurnal changes in electrical properties. Neurotransmitters and neuromodulators were applied focally with a micropipette to their SCN projection sites; effects on the phase of the electrical activity rhythm were determined from the behavior of the ensemble of single units. In addition, the levels of glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the inhibitory neurotransmitter γ-aminobutyric acid [GABA]) in SCN micropunched from brain slices were assessed over the circadian cycle by Western blotting. In experiments aimed at understanding regulation by retinohypothalamic afferents, Dr. Michael Rea's laboratory has examined several parameters after stimulation of the optic nerve (the release of excitatory amino acids, field potential activity, and sensitivity to pharmacological blockade of SCN *in vitro*); these results are discussed in the paper by Rea and colleagues in this issue.

Hypotheses tested in our research include the following: (1) Pacemaking properties are distributed throughout the SCN; (2) the neurons of the SCN are homogeneous with respect to their electrical and pacemaking properties; (3) neuromodulators from inputs implicated in phase shifts of behavior by dark pulses (serotonin [5-HT] from the raphe, neuropeptide Y [NPY] from the intergeniculate leaflet) are effective phase-shifting agents for SCN during the circadian day; (4) GAD levels are constant over the circadian cycle; and (5) light information carried by the retinohypothalamic tract affects the SCN via excitatory amino acids (viz., glutamate). Our progress toward evaluating these hypotheses is presented here.

Regarding the first hypothesis, cellular organization of the SCN pacemaker was examined by determining whether regional variation in the circadian oscillation in ensemble neuronal activity was expressed in the intact SCN brain slice. Post hoc analysis of the pattern of activity recorded on day 2 *in vitro* revealed that, indeed, both the ventrolateral (VL) and dorsomedial (DM) subregions of the SCN exhibited pronounced activity peaks (Tcheng et al., 1989). These were not apparently altered by bisecting the brain slice at the base of the third ventricle, which severs connections between the bilaterally paired SCN. In other words, each SCN appears to be an autonomous pacemaker.

The rat SCN pacemaker has two natural anatomical subdivisions: (1) the VL SCN, site of the relatively larger neurons (mean diameter 9.6 ± 1.5 μm), including those containing vasoactive intestinal peptide (VIP) upon which afferents from the retina, intergeniculate leaflet, and dorsal raphe form synapses (van den Pol and Tsujimoto, 1985; Guy et al., 1987); and (2) the DM SCN, which is composed of relatively smaller vasopressin-containing neurons (mean diameter 7.8 ± 0.9 μm) that give rise to numerous efferent fibers (van den Pol, 1980). We hemisected each SCN so as to separate the VL SCN from the DM SCN, in order to determine the pacemaking ability of each region. When activity in a single intact SCN was monitored continuously for 32 hr, the characteristic sinusoidal circadian pattern in firing frequency (Gillette and Prosser, 1988) was observed with high-amplitude peaks at 24-hr intervals (Gillette et al., 1992). Surprisingly, hemisection did not affect this pattern in the VL SCN, whereas amplitude and shape of the neuronal activity rhythm appeared altered in

the DM SCN after surgical isolation (Tcheng and Gillette, 1990; Gillette et al., 1992). These preliminary results are consistent with the alternative to the hypothesis tested—namely, that there may be localization of pacemaking function within the SCN.

In order to address the second hypothesis (the issue of neuronal heterogeneity at the cellular level), we have begun to examine individual neurons of the SCN, using the whole-cell patch recording technique (Blanton et al., 1989) in the rat brain slice. Initial efforts were concentrated in the VL SCN, although we have recently begun to extend our observations to the DM SCN. By using this approach, we have found the SCN to be composed of a variety of electrophysiologically distinct cell types (Gallman et al., 1991; Gallman and Gillette, 1993). Our observations challenge the hypothesis, which had been suggested by early workers in the field (Wheal and Thompson, 1984), that the SCN is electrically homogeneous. Furthermore, preliminary findings concerning DM SCN neurons do not contradict the alternative hypothesis—that there *are* regional differences in properties of neurons of the VL and DM SCN.

The third hypothesis concerns the potential role of 5-HT and NPY in mediating phase shifts induced by behavioral arousal and dark pulses, respectively. This was tested by applying a 30-fl droplet of either 10^{-6} M 5-HT or NPY to the VL SCN in brain slices. Effects upon the rhythms of neuronal activity of 5-min microdrop applications at various points in the circadian cycle were assessed over 1–2 days posttreatment. These experiments were designed to evaluate the permanence, receptor specificity, and dose dependency of phase changes, compared to the responses of controls treated with microdrops of medium. SCN sensitivity to 5-HT was restricted to the subjective day (circadian times 2–11 [CTs 2–11], with peak phase advance occurring at CT 7 (Fig. 1). Both 5-carboxyamidotryptamine (5-CT), a 5-HT_1 receptor agonist, and 8-hydroxy-2-*n*-propylamino)-tetralin (8-OH-DPAT), an agonist specific for the 5-HT_{1A} receptor subtype, also induced large advances in the peak

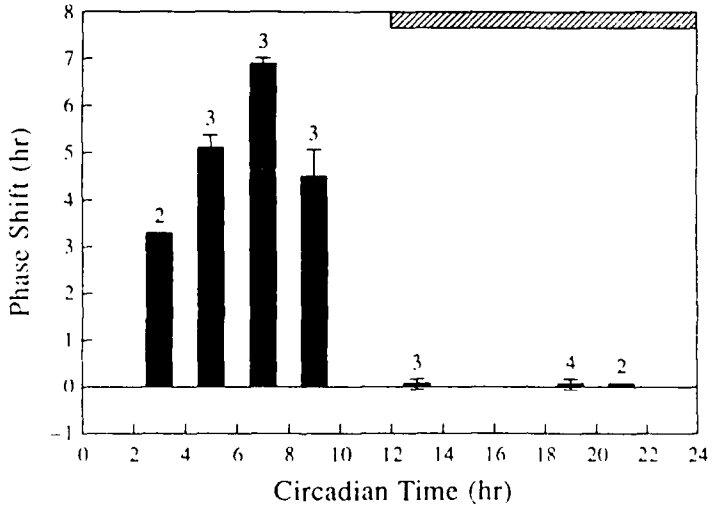

FIGURE 1. Phase response curve relating the time of 5-HT treatment to the time of appearance of the peak in ensemble neuronal firing rate in the next circadian cycle. For treatments at CT 7, the time of maximal phase advance, activity was monitored for 2 days posttreatment. The peak on day 3 appeared at CT 0, 24 hr after that on day 2, demonstrating that the phase shift measured on day 2 was complete. The hatched horizontal bar represents subjective night. Adapted from Medanic and Gillette (1992a.)

of the electrical activity rhythm in SCN *in vitro*; these treatments were without effect during the subjective night (Medanic and Gillette, 1992a). Thus, the regulatory effect of 5-HT on the neurons of the VL SCN appears to be mediated through a 5-HT_{1A} receptor subtype. Interestingly, the SCN in slices shows both late-day and late-night windows of sensitivity to NPY-induced phase resetting (Medanic and Gillette, 1993). The daytime period of sensitivity to NPY is distinct from, but overlaps with, the latter portion of 5-HT sensitivity (Medanic and Gillette, 1992b). These results are consistent with roles for 5-HT and/or NPY in nonphotic (Mrosovsky and Salmon, 1987) and/or dark-pulse (Boulos and Rusak, 1982; Ellis et al., 1982; Albers and Ferris, 1984) entrainment processes.

Next, we examined the hypothesis that levels of GAD remain constant over the circadian cycle. GAD is the biosynthetic enzyme for the most abundant inhibitory neurotransmitter in the SCN, GABA. Between 50% and 100% of SCN neurons are GAD-positive (van den Pol and Moore, personal communication). GABA-ergic neurons, those containing GAD, are distributed throughout the nucleus (van den Pol and Tsujimoto, 1985), and GABA administration inhibits 65% of SCN neurons (Liou et al., 1990). The amplitude of the daily oscillation of mean spontaneous firing frequency for rat SCN neurons shows an excursion between 8 Hz at midday and 2 Hz at midnight. This may be regulated, at least in part, by changing GABA levels, which in turn would be controlled by changing GAD levels and/or GAD activity over the course of the circadian cycle. To enable us to evaluate this issue, SCN from brain slices maintained *in vitro* were rapidly frozen on dry ice, and the SCN were removed by micropunch. Western blot analysis of the two major GAD isozymes, GAD_{65} and GAD_{67}, demonstrated that both were present in SCN at the four 6-hr intervals examined, and that the levels underwent a significant circadian variation (Richard et al., 1991). Whether circadian modulation of GAD activity also affects GABA biosynthesis in the SCN is currently under investigation.

The fifth hypothesis tested in this research regards the potential role of excitatory amino acids in mediating the effects of light at night in this system. These experiments have been addressed primarily by Dr. Michael Rea's laboratory, as discussed in Rea et al.'s paper in this issue. Our laboratory has begun to evaluate the effect on the circadian rhythm of ensemble neuronal activity of the excitatory amino acid glutamate (GLU). GLU (at 10^{-2} M in a 1-μl droplet of medium) was applied for 10 min to one SCN *in vitro*. Preliminary results suggest that this brief GLU application can induce both phase delays and advances at night, and strengthens the possibility that GLU may mediate the phase-shifting effects of light on the SCN pacemaker.

With the finding that pacemaking properties reside in the VL SCN (at least), we can proceed to focus upon the electrophysiological properties of this region. Differences between the VL and DM SCN will be interesting to document. Circadian periods of sensitivity to 5-HT, NPY, and GLU, tentatively described in this report, differ from those we have described for cyclic adenosine monophosphate (Prosser and Gillette, 1989), cyclic guanosine monophosphate (Prosser et al., 1989) and melatonin (McArthur et al., 1991), emphasizing the complexity of SCN regulatory processes. Because the SCN integrate most circadian behaviors and metabolic fluxes, this research has basic relevance to understanding circadian dysfunction induced by transmeridian travel and sustained irregular work schedules, with application to improving human performance under conditions that induce circadian desynchronization.

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SEROTONIN REGULATES THE PHASE OF THE RAT SUPRACHIASMATIC CIRCADIAN PACEMAKER *IN VITRO* ONLY DURING THE SUBJECTIVE DAY

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SUMMARY

1. The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary pacemaker for circadian rhythms in mammals. The 24 h pacemaker is endogenous to the SCN and persists for multiple cycles in the suprachiasmatic brain slice.

2. While serotonin is not endogenous to the SCN, a major midbrain hypothalamic afferent pathway is serotonergic. Within this tract the dorsal raphe nucleus sends direct projections to the ventrolateral portions of the SCN. We investigated a possible regulatory role for serotonin in the mammalian circadian system by examining its effect, when applied at projection sites, on the circadian rhythm of neuronal activity in rat SCN *in vitro*.

3. Eight-week-old male rats from our inbred colony, housed on a 12 h light : 12 h dark schedule, were used. Hypothalamic brain slices containing the paired SCN were prepared in the day and maintained in glucose and bicarbonate-supplemented balanced salt solution for up to 53 h.

4. A 10^{-11} ml drop of 10^{-6} M-serotonin (5-hydroxytryptamine (5-HT) creatinine sulphate complex) in medium was applied to the ventrolateral portion of one of the SCN for 5 min on the first day *in vitro*. The effect of the treatment at each of seven time points across the circadian cycle was examined. The rhythm of spontaneous neuronal activity was recorded extracellularly on the second and third days *in vitro*. Phase shifts were determined by comparing the time-of-peak of neuronal activity in serotonin- *vs.* media-treated slices.

5. Application of serotonin during the subjective day induced significant advances in the phase of the electrical activity rhythm ($n = 11$). The most sensitive time of treatment was CT 7 (circadian time 7 is 7 h after 'lights on' in the animal colony), when a $7{\cdot}0 \pm 0{\cdot}1$ h phase advance was observed ($n = 3$). This phase advance was perpetuated on day 3 *in vitro* without decrement. Serotonin treatment during the subjective night had no effect on the timing of the electrical activity rhythm ($n = 9$).

6. The specificity of the serotonin-induced phase change was assessed by treating

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slices in the same manner with a microdrop of serotonergic agonists, 5-carboxamidotryptamine, that targets the 5-HT_1 class of receptors, or 8-hydroxy-dipropylaminotetralin (8-OH DPAT), that acts on the 5-HT_{1A} receptor subtype. Daytime (CT 9) application of 5-carboxamidotryptamine resulted in a $6{\cdot}0 \pm 0{\cdot}1$ h phase advance ($n = 3$), while treatment during the subjective night (CT 15, $n = 2$) had little observable effect. Similarly, treatment with 8-OH DPAT at CT 9 induced a phase advance of $6{\cdot}9 \pm 0{\cdot}1$ h ($n = 3$) in the rhythm of electrical activity.

7. These results demonstrate that serotonin can induce large phase changes in the SCN circadian pacemaker and that the SCN undergoes endogenous changes in sensitivity to serotonin. The data suggest that serotonergic inputs to the ventrolateral SCN can regulate the circadian pacemaker during the daytime.

INTRODUCTION

Substantial evidence points to the suprachiasmatic nuclei (SCN) as the endogenous pacemaker that regulates the timing of most circadian rhythms of behaviour, physiology and metabolism in mammals. The SCN generate circadian patterns of neural activity (Inouye & Kawamura, 1979) and glucose utilization (Schwartz & Gainer, 1977; Schwartz, Davidsen & Smith, 1980) in various brain regions *in vivo*. Near-24 h rhythms continue in surgically isolated SCN but are abolished in other brain regions by deafferentation from SCN (Inouye & Kawamura, 1979). The nuclei are capable of sustaining rhythmicity of endogenous neuronal firing (Gillette & Prosser, 1988) and peptide secretion (Earnest & Sladek, 1987) for multiple cycles *in vitro*, under constant conditions devoid of any external time cues.

Green & Gillette (1982) showed that the endogenous circadian rhythm of electrical activity (measured by extracellular recording) present in SCN of intact organisms is preserved in SCN within a hypothalamic brain slice. The waveform and the time-of-peak in this oscillation were found to be unaffected by day-time preparation of brain slices (Gillette, 1986; Gillette & Reppert, 1987). The robustness, uniformity and stability of the waveform over multiple cycles *in vitro* permit use of this oscillation to monitor the underlying activity of the pacemaker in isolation from inputs and modifiers (Prosser & Gillette, 1989). We used this *in vitro* preparation to examine regulation of circadian timing by exogenous serotonin.

Photic changes in the external world are primary regulators of circadian timing. The retino-hypothalamic tract and the geniculo-hypothalamic tract form the visual pathways of entrainment. The retino-hypothalamic tract carries light information directly from the retina to the ventrolateral portion of the SCN (Moore & Lenn, 1972). Although the transmitter in the retino-hypothalamic tract has not been completely identified, it is thought to influence the SCN via the excitatory amino acid glutamate (Liou, Shibata, Iwasaki & Ueki, 1986; Cahill & Menaker, 1987). The geniculo-hypothalamic projection forms a secondary visual input from the lateral geniculate nucleus to the SCN (Swanson, Cowan & Jones, 1974; Card & Moore, 1982). This tract also relays information about the lighting regime, but is thought to be involved in mediating the effects of dark pulses (Harrington & Rusak, 1988). Terminals of this neuropeptide Y-containing tract are overlapping those of the retinal pathways (Card & Moore, 1982; Groos, Mason & Meijer, 1983).

Projections from the midbrain raphe nuclei form a distinctive pathway leading to

the SCN. While the functional nature of these projections has not been elucidated, it can be hypothesized that they play a modulatory role. The raphe projects to the ventrolateral portions of the rostral SCN (Aghajanian, Bloom & Sheard, 1969; van den Pol & Tsujimoto, 1985), as well as to the ventral lateral geniculate nucleus (Moore, Halaris & Jones, 1978). Its serotonergic terminals in the SCN overlap with those directly regulated by photic stimulation, the retino-hypothalamic and geniculo-hypothalamic inputs (Ueda, Kawata & Sano, 1983; Guy, Bosler, Dusticier, Pelletier & Calas, 1987). The pathway from the raphe to the SCN is thought to be activated by arousal, possibly through motor activity stimulated by the onset of darkness (Mrosovsky & Salmon, 1987).

Serotonin is a widely distributed neurotransmitter that in many cases causes suppression of spontaneous firing rates of neurons. This is true in the case of the SCN. Stimulation of the raphe *in vivo* results in an overall inhibition of firing rates of SCN neurons (Groos *et al.* 1983). Similarly, microionophoretic application of serotonin to the SCN has an inhibitory effect on the firing rate of the majority of neurones, both *in vitro* and *in vivo* (Mason, 1986; Meijer & Groos, 1988).

Interestingly, SCN neurons in brain slices show a circadian variation in sensitivity to serotonin in terms of firing rate, such that the neurons are more sensitive to inhibition of firing rate by serotonin in the subjective night (Mason, 1986). Besides the circadian rhythm of sensitivity to serotonin, the SCN also display a circadian rhythm in recovery from serotonin. Mason (1986) demonstrated that the time of recovery after inhibition of neuronal activity was longer when serotonin was administered during the day-time than during the night.

Serotonin receptors are abundantly distributed throughout the SCN (van den Pol & Tsujimoto, 1985). These receptors are capable of serotonin reuptake in a temporally dependent manner, with higher rates of reuptake during the subjective night (Meyer & Quay, 1976). Intragastric administration of imipramine, a serotonin reuptake blocker, was found to lengthen and enhance the inhibitory action of serotonin in the SCN (Meijer & Groos, 1988). Studies by Wirz-Justice, Krauchi, Morimesa, Willener & Feer (1983) demonstrated that serotonin receptors in the SCN have a temporal sensitivity to imipramine binding which peaks during the night phase in the rat.

Serotonergic inputs are not required for sustaining endogenous circadian organization of the pacemaker. Application of 5,7-dihydroxytryptamine, which selectively lesions serotonergic inputs, does not affect the period of oscillator *in vivo* (Honma, Watanabe & Hiroshige, 1979; Smale, Michels, Moore & Morin, 1990). Also complete lesions of the raphe have little effect on the entrainability of the animal by light (Kam & Moberg, 1977). However studies on activity-stimulated phase shifts of locomotor activity in the day (Mrosovsky & Salmon, 1987) and the circadian nature of serotonin sensitivity in the SCN suggest possible involvement of serotonin in day-time regulation of the circadian pacemaker.

In this study we directly tested the hypothesis that serotonin has a regulatory role in the SCN. Serotonin was briefly and focally applied to the region of raphe inputs to the rat SCN in brain slices. Effects of serotonin application at different phases of the circadian cycle were determined by measuring the rhythm of neuronal activity on the second and third day after treatment. The specificity of the serotonin-induced phase shifts was assessed by treatment with agonists 5-carboxamidotryptamine and

8-hydroxy-dipropylaminotetralin (8-OH DPAT), which are specific for 5-HT_1 and 5-HT_{1A} receptor subtypes, respectively. We found that serotonin has a strong phase-shifting effect in the middle of the day, but has no effect when applied during the night.

METHODS

Preparation of brain slices

Male Long–Evans rats from our inbred colony were used in this study. Animal care and brain slice preparation were performed humanely, in accordance with guidelines from the American Veterinary Medical Association. The animals were kept on a schedule of 12 h of light, and 12 h of darkness, with food and water available *ad libitum* from birth to 8 weeks, when they were studied. The animals were killed during the 'lights on' period of the 24 h cycle, 1–10 h before serotonin application. This was necessary to avoid phase-shifting effects which have been shown to occur with manipulations in the night-time (Gillette, 1986). The animals were gently introduced into the guillotine, decapitated, and the brain was quickly dissected from the skull. The brain was then manually sectioned to form a block of tissue containing the hypothalamic region. This block of tissue was transferred to a mechanical tissue chopper where 500 μm in coronal slices were made. The hypothalamic slices containing the SCN (clearly visible under the microscope) were reduced even further under the microscope by cutting away excess hypothalamic tissue, and then transferred to the brain slice dish where they were maintained for up to 3 days. This procedure was performed within 5 min from the time of decapitation to avoid development of irreversible hypoxic conditions. A diagram of the hypothalamic brain slice used in this study can be seen in Fig. 1.

The brain slice dish, consisting of an inner and an outer chamber, was modelled after Hatton, Doran, Salm & Tweedle (1980). The outer chamber, a water-bath that provides a constant environment for the slices, was filled with double-distilled water warmed to 37 °C and bubbled with 95% O_2, 5% CO_2 to provide a moist, high-oxygen atmosphere at the surface of the slice. The inner chamber was made up of a central and an outer well that were continuously perfused with supplemented salt solution at a rate of 30 ml/h. The medium consisted of Earle's Balanced Salt Solution (0·2 g/l $CaCl_2$, 0·4 g/l KCl, 0·0977 g/l $MgSO_4$, 6·8 g/l NaCl and 0·14 g/l $NaH_2PO_4.H_2O$; GIBCO), supplemented with 24·6 mM-glucose and 26·2 mM-$NaHCO_3$, warmed in a reservoir to 39 °C at a pH of 7·20 and oxygenated by a gas mixture of 95% O_2 and 5% CO_2 (which adjusted the pH at 7·40). The temperature of the medium around the brain slices in the central well was 37 ± 0·1 °C.

The slices were placed on a fibre mesh (Mr Coffee® filter) that covered the central well of the inner chamber of the dish. The medium level was adjusted to come up and around the tissue to bathe the slices without floating them; the slices rested at the interface of the medium and the atmosphere. The tissue was illuminated by a fibre-optics lamp throughout the experiment.

Experimental treatments

Serotonin (5-hydroxytryptamine creatinine sulphate, Sigma, USA) or serotonergic agonists, 5-carboxamidotryptamine (RBI) and 8-hydroxy-dipropylaminotetralin (8-OH DPAT, RBI), were applied to the ventrolateral portion of one of the paired SCN in the slice (Fig. 1), for 5 min at various times across the circadian cycle. Fifteen minutes before the treatment, a 10^{-6} M-solution of serotonin in supplemented salts medium was prepared, and warmed and oxygenated for ~ 2 min. Silanized micropipettes (tip = 2 μm) were smoothed by fire-polishing the large end, back-filled with the 10^{-6} M-serotonin solution, and fitted into the end of 1 m length of Teflon tubing. The tubing was filled with distilled water and attached to a syringe. The micropipette connected to this delivery apparatus was fitted on a micromanipulator and advanced, under microscopic guidance, until it was above the place of delivery, on the ventrolateral SCN. Perfusion of medium throughout the chamber was stopped. A microdrop was created by pressing down on the syringe plunger to form a drop 3–4 μm in diameter, and then gently pulling back to prevent the drop from enlarging further. To deliver the microdrop the micropipette was advanced until the drop made contact with the surface of the slice. After 5 min, the slice was manually rinsed with medium in a direction away from the rest of the SCN (see Fig. 1).

Test runs prior to the actual experiments were performed with 0·1% Methylene Blue in medium to determine the extent of drop spreading. These drops were confined to a small area within the ventrolateral region of the SCN. The estimated volume of a typical microdrop was 3×10^{-11} ml.

This was calculated from the volume of a spherical drop with a 3–4 μm diameter. This diameter was directly measured by an ocular micrometer on test drops generated with a similar pressure to that in actual experiments, from silanized micropipettes with 2 μm tips, and is representative of the size of experimental microdrops. When the microdrop touches the surface of the slice, the serotonin becomes diluted immediately. Thus, the effective concentration of serotonin at the receptor sites is considerably less than 10^{-6} M, but cannot be determined directly.

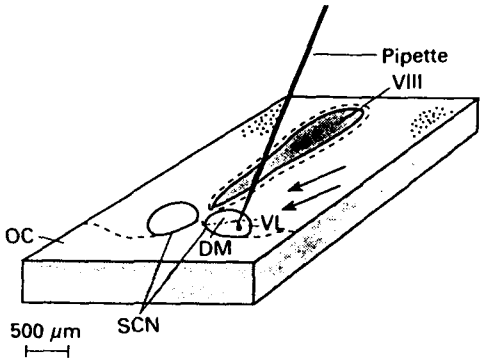

Fig. 1. Diagrammatic illustration of the microdrop application technique on a 500-μm-thick coronal hypothalamic brain slice. The suprachiasmatic nuclei (SCN) are located on either side of the third ventricle (VIII) and dorsal to the optic chiasm (OC). The micropipette is positioned directly above the ventrolateral region of one SCN (VL denotes ventrolateral, and DM dorsomedial). Arrows indicate the direction of media flow rinsing the serotonin treatment off the slice.

Agonists were similarly applied, at 10^{-6} M concentrations, with the only procedural difference being that the 8-OH DPAT solution was made in unoxygenated solution due to the instability of this agonist in oxygen.

To verify that the observed phase shifts were due to serotonin treatment rather than the mechanical or thermal perturbations during treatment, experimental treatments were compared with controls in which a microdrop of medium alone was applied in the same way as in the experiments, to the ventrolateral portions of one of the SCN in a brain slice.

Electrical recording technique

In order to distinguish the effects of the treatment on the pacemaker from acute effects on neuronal activity, extracellular recordings of spontaneous neuronal activity were initiated with the onset of what would be the subjective day in the rat, on the second and third day *in vitro*. This was 11–50 h after application of the microdrop of serotonin to the SCN. We have previously established that the SCN in brain slice generates a complete sinusoidal oscillation in the firing rate of the ensemble of neurons (Gillette, 1986). To facilitate recording for long periods, only that portion of the day surrounding the expected and anticipated peak was studied.

A silver chloride-coated ground wire was placed through the filter mesh, while glass microelectrodes (tip = 2 μm), back-filled with 5 M-NaCl, were used to record electrical activity. The recording electrode was fitted in a micromanipulator and positioned over the slice so that it was in contact with the surface of the tissue. A Narashige MO-8 hydraulic microdrive was used to further advance the electrode through the tissue. The signal picked up by the recording electrode was amplified, filtered (bandpass, 200–2000 Hz) and displayed on a Tetronix oscilloscope, using a WPI-121 window discriminator to isolate single cell activity. A signal-to-noise ratio of 2:1 was the minimum requirement for discriminating a cell. The single-cell activity was recorded by a spike-frequency counting program on a Commodore computer. Cells were monitored for two 120 s trials, where the firing rates of 10 s bins were averaged to determine the mean firing rate of the unit. On average, four to six cells were sampled per hour, with a recording time of 8–12 h providing a total of forty to seventy units of data with which to assess the SCN electrical activity rhythm for each peak studied. Neuronal activities were sampled at random throughout the SCN. Previous studies have shown that the SCN functions as a coherent pacemaker with a uniform rhythm of electrical

activity measurable in both dorsomedial and ventrolateral regions (Tcheng, Gillette & Prosser, 1989). The recorded firing rates of individual cells were averaged together and 2 h means, with 15 min lags, were calculated and plotted. The time-of-peak, defined as the circadian time at which the 2 h mean of the firing rates of the sampled cells reached a symmetrical maximum, was determined. The experimental time-of-peak was compared to microdrop controls in order to determine the magnitude of the phase shift induced by the experimental treatments.

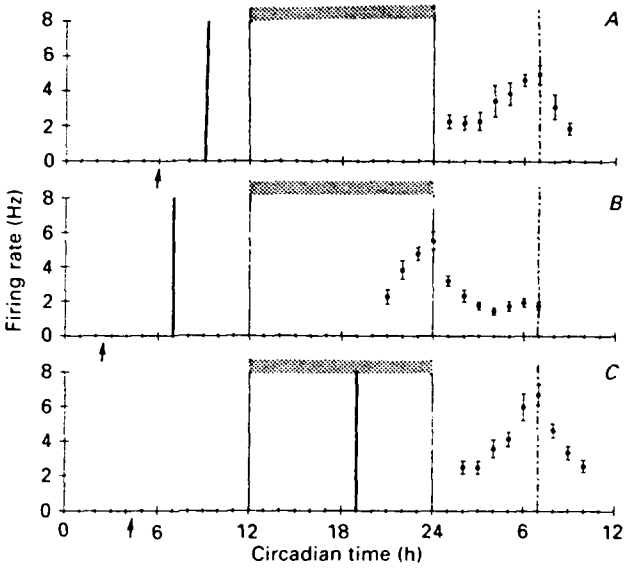

Fig. 2. The SCN sensitivity to phase shifting by serotonin changes over the course of the day. *A*, slices treated with a microdrop of medium at CT 9 on day 1. Rhythm of endogenous neuronal activity on day 2 peaked at CT 6·9. *B*, slice treated with a microdrop of 10^{-6} M-serotonin at CT 7 on day 1. The peak in the rhythm of electrical activity on day 2 was phase advanced 7 h to CT 0. *C*, slice treated at CT 19, on day 1. The peak occurred at CT 7 on day 2 which is overlapping with the peak in the rhythm of control slices. ● represent the 2 h means ± S.E.M., plotted with 1 h lags, of the recorded neuronal activity rhythm on the second day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in untreated slices. The horizontal stippled bar indicates the time of the donor's subjective night in the colony. Arrows point out the time of slice preparation.

RESULTS

Control experiments

Because the time-of-peak of electrical activity in the SCN is highly predictable between experiments and stable over time *in vitro* (CT 6·9 ± 0·2, $n = 8$, Prosser & Gillette, 1989), as well as easy to visually discern, we use it to mark the phase of the underlying circadian pacemaker. In the present series of experiments, the time-of-peak for untreated SCN of rats from our inbred colony occurred at CT 6·9 ± 0·1 ($n = 3$), which is in agreement with previously reported results. In the microdrop controls, applied at CT 9, the time-of-peak was at CT 6·9 ± 0·1 ($n = 3$, Fig. 2*A*), which is identical to the established peak times for untreated SCN. This demonstrates that the microdrop technique did not, in itself, cause phase shifts.

Serotonin experiments

We found that serotonin can affect the SCN pacemaker. Single-unit recordings of the population of neurons revealed that when a microdrop of serotonin was administered in mid-subjective day on the first day *in vitro*, the peak of the next

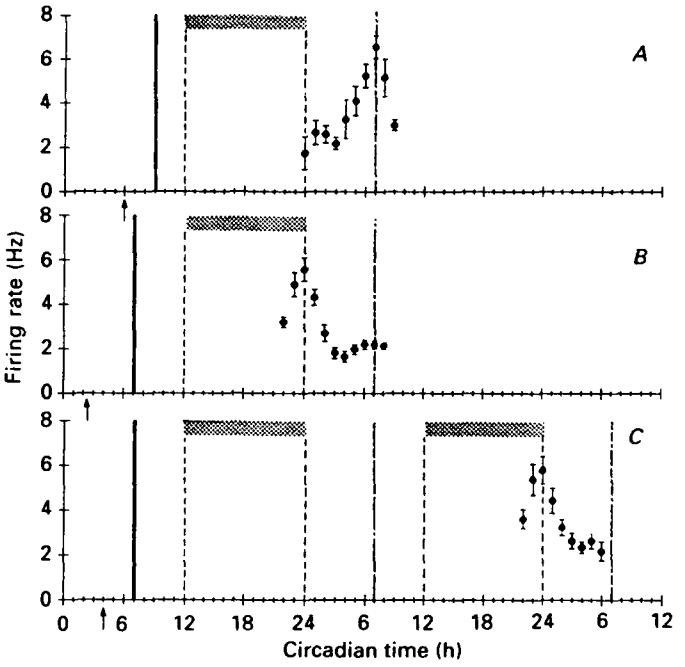

Fig. 3. Serotonin induced permanent phase shifts *in vitro*. *A*, rhythm of endogenous neuronal activity recorded on day 2 after treatment with medium on day 1. *B*, localized application of serotonin to the ventrolateral region of the SCN slice at CT 7 resulted in a 7·0 h phase advance in the rhythm of electrical activity on day 2. *C*, recording on day 3, in a separate experiment, following treatment with serotonin at CT 7 on day 1, indicated a 7·0 h phase advance. This is overlapping with the mean phase advance seen on day 2. ● represent the 2 h means ± S.E.M. of the recorded neuronal activity rhythm on the second and third day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in control slices. The horizontal stippled bar indicates the time of the donor's night in the colony. Arrows point out the time of slice preparation.

day's rhythm of electrical activity was significantly advanced. After a 5 min application of serotonin at CT 7 on day 1, the time-of-peak occurred at CT 23·9 ± 0·1 ($n = 3$) on day 1, rather than at CT 6·9 ± 0·1, the time-of-peak on day 2 in control slices (Fig. 2*B*). This is a 7·0 ± 0·1 h advance in the neuronal activity rhythm. Statistical analysis of the data using Student's *t* test, which compared the time-of-peak in serotonin-treated slices to that of control confirms that the effect of serotonin at CT 7 is significant ($P < 0·001$). In a separate experiment, the electrical activity rhythm was recorded on day 3 after serotonin treatment on day 1 at CT 7 (Fig. 3). The peak appeared at CT 0, 23·9 h after the peak on day 2, and still advanced in

phase by ~7 h. These results indicate that the phase change due to serotonin treatment at CT 7 on day 1 is a permanent one.

Administration of serotonin in the subjective night, however, had no apparent effect on the rhythm of neuronal activity on the second day. After exposure of the

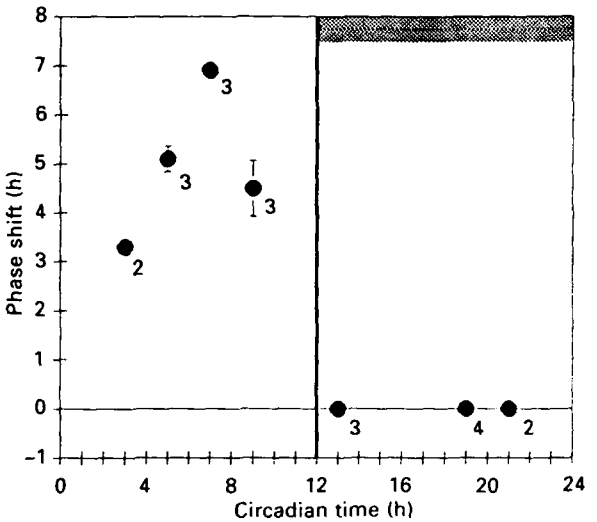

Fig. 4 Phase-response curve for serotonin. The x-axis denotes circadian time (CT) of treatment (h) and the y-axis indicates the average magnitude and direction of the serotonin induced phase shift (h). The magnitude of the shift of the time-of-peak of the electrical activity rhythm was determined in relation to time-of-peak in slices treated with medium. ● represent the mean ± S.E.M. phase shift. The subscript number is the number of experiments performed at a particular time point. The vertical bar denotes the time of 'lights off' in the colony and the horizontal stippled bar indicates the night.

SCN to serotonin at CT 19, the peak in activity on the next day was at CT 6·7 ± 0·1 ($n = 3$, Fig. 2*C*). This time overlaps with controls.

Similar measurements were made after a microdrop of serotonin was applied at one of three other time points during the subjective day, CT 3 ($n = 2$), 5 ($n = 3$), 9 ($n = 3$), and two other points during the subjective night, CT 13 ($n = 3$), and 21 ($n = 2$). A phase-response curve relating the circadian time of serotonin treatment to its effect on the phase of the electrical activity rhythm is seen in Fig. 4. Treatment with serotonin in the subjective day-time resulted in robust, persistent phase advances in the rhythm of neuronal activity, while it had no effect on the pacemaker in the subjective night.

Agonist experiments

5-Carboxamidotryptamine

Treatment of slices with 5-carboxamidotryptamine, a serotonergic agonist specific for the 5-HT_1 receptor subtype (Peroutka, 1988), resulted in time-dependent phase shifts in the rhythm of neuronal activity. When administered during the day at CT 9, a time when serotonin induced a 4·6 h phase advance, 5-carboxamidotryptamine

caused a 6·0±0·1 phase advance ($n = 3$, Fig. 5*A*). Conversely, treatment of SCN at CT 15 had little effect on the phase of the rhythm of electrical activity (Fig. 5*C*). The time-of-peak was recorded at CT 6·5 ($n = 2$) which is near the control peak time.

8-OH DPAT

Treatment at CT 9 with 8-OH DPAT, an agonist specific for the 5-HT_{1A} receptor subtype (Middlemiss & Fozard, 1983), also resulted in significant phase shifts in the

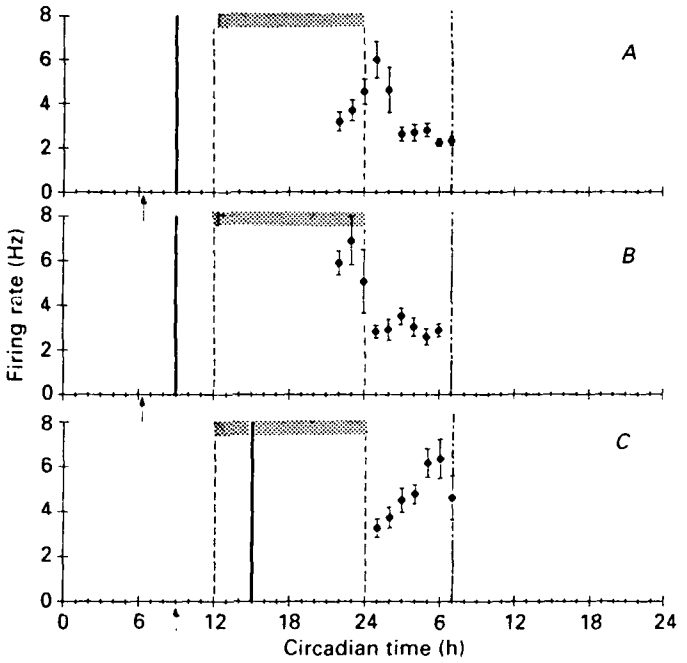

Fig. 5. Serotonergic agonists phase-shift the rhythm of electrical activity. *A*, slice treated at CT 9 on day 1 with 5-carboxamidotryptamine. The time-of-peak on day 2 occurred 6·5 h earlier than in control slices. *B*, slice treated at CT 9 on day 1 with 8-OH DPAT. The peak in the rhythm of electrical activity, recorded on day 2 was advanced by 8 h. *C*, slice treated on day 1 with 5-carboxamidotryptamine at CT 15. The time-of-peak was recorded at CT 6·25 which is overlapping with the range of peak times in control slices. ● represent the 2 h means ± S.E.M. of the recorded neuronal activity rhythm on the second and third day. The vertical bar indicates the time of serotonin treatment and the interrupted line indicates the time-of-peak observed in slices treated with medium. The horizontal stippled bar indicates the time of the donor's night in the colony. Arrows point out the time of slice preparation.

rhythm of neuronal activity. The time-of-peak was observed at CT 0·0±0·1 ($n = 3$), indicating a 7 h phase advance (Fig. 5*B*).

DISCUSSION

Our results on the suprachiasmatic slice preparation demonstrate that serotonin can act directly on the SCN to change the phase of the pacemaker. The responses at the time points tested indicate that SCN sensitivity to serotonin released at raphe

projection sites is limited to the subjective day of the circadian cycle. Serotonin treatment on day 1 *in vitro* phase advances the rhythm of neuronal activity within the next 12 h so that the peak appears early on the second and third day. The stable phase relationship between days 2 and 3 suggests that the pacemaker has been permanently reset by the brief (5 min) exposure to serotonin. The changes in the phase of the pacemaker were also shown to be serotonin specific, as demonstrated by the agonist-induced phase shifts.

Analysis of SCN in the brain slice, where it is isolated from modulating inputs and feedback loops from other brain regions, permits direct assessment of properties intrinsic to the pacemaker. This approach therefore provides insight to the basic characteristics of the system. The present study suggests that serotonin can induce 7 h phase changes in the pacemaker, should the other modulatory pathways be absent. While such large phase shifts are not uncommonly observed in the slice preparation (cyclic AMP induces 5 h phase shifts, Prosser & Gillette, 1989; K^+ induces 6 h phase shifts, Gillette, 1987), shifts of this magnitude have rarely been reported *in vivo*, where the magnitude of the effect of a 1 h stimulus does not exceed 2 h. In addition to the difference in magnitude, phase shifts *in vitro* differ in rate. We have not observed transient changes in the phase of the rhythm of electrical activity over successive days *in vitro* (Gillette, 1987). Comparison of data sets indicates that the pacemaker can be reset directly and rapidly *in vitro*, but that *in vivo* it is modified by numerous modulatory influences and feedback loops such that the phase shifts are damped in magnitude and rate (as in DeCoursey, 1964). From the massive size of the serotonin phase advance relative to day-acting zeitgebers *in vivo* it follows that potent inertial forces normally act to slow the size and rate of the change. However, the fact that this is one of the largest phase advances that we have found among agents studied *in vitro* may also be a reflection of the strength of this pathway relative to other modulatory forces acting in the same time domain.

While the effect of serotonin injection into the SCN *in vivo* has not yet been examined, indirect evidence suggests that serotonergic pathways may stimulate phase shifts in behaviour in the animal during the day-time. Forced activity in the middle of the day facilitates entrainment of mammals to novel light–dark schedules (Mrosovsky & Salmon, 1987). Hamsters that were confined to a running wheel after being exposed to a phase-altered lighting schedule re-entrained more than twice as rapidly as undisturbed animals. Further experiments indicated that hamsters undergo accelerated phase shifting by behavioural arousal, by forced activity or social interaction at midday (Mrosovsky, 1988), a time when serotonin induces phase shifts *in vitro*. The physiological mechanism by which non-photic signals entrain the SCN has not yet been determined. Mrosovsky (1988) has hypothesized that state of arousal is communicated to the SCN via serotonergic projections from the raphe nuclei. Our data lend support to this notion.

Interestingly, the SCN period of sensitivity to serotonin overlaps the period of sensitivity to cyclic AMP. Application of cyclic AMP analogues resulted in phase advances in the rhythm when applied in the subjective day (CT 3–11) with a peak sensitivity between CT 5 and 7; treatment in the subjective night did not significantly affect the rhythm of electrical activity (Gillette & Prosser, 1988). Similarly, administration of substances that elevate the endogenous levels of cyclic AMP

(phosphodiesterase inhibitors that prevent cyclic AMP breakdown or stimulators of adenylate cyclase) altered the phase of the rhythm of neuronal activity in a similar manner (Prosser & Gillette, 1989).

Extensive research on the organization of the endogenous pacemaker in the eye of the mollusc *Aplysia* has led to the conclusion that serotonin plays a regulatory role in that circadian pacemaker. *In vitro* experiments with the *Aplysia* ocular pacemaker (Corrent, Eskin & Kay, 1982) have demonstrated that serotonin acts as a strong phase-shifting agent through a sequence of events separate from the light entrainment pathway. Bath applications of serotonin to preparations of the isolated eyes of *Aplysia*, for a minimum pulse of 1·5 h, resulted in phase advances of 3–4 h between CT 5 and 11 and phase delays of 2·5 h from CT 22 to CT 2 (Corrent, Meadoo & Eskin, 1978).

Phase shifts similar to those induced by serotonin were stimulated by cyclic AMP and its analogues (8-benzylthio-cyclic AMP) in the *Aplysia* pacemaker (Eskin, Corrent, Lin & Meadoo, 1982). In addition, administration of phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine, IBMX, RO20-1724 and papaverine) that block cyclic AMP breakdown resulted in serotonin-like phase-shifted rhythms (Eskin *et al.* 1982). When the phosphodiesterase inhibitor was administered simultaneously with serotonin, no augmentation was seen suggesting that cyclic AMP and serotonin were acting through the same pathway (Eskin *et al.* 1982). Additional support for this proposition comes from the finding that serotonin produces changes in the endogenous levels of cyclic AMP in the eye of *Aplysia* (Eskin *et al.* 1982). Further steps in the serotonin pathway have also been studied. The protein synthesis inhibitor, anisomycin, blocks serotonin phase-shifting after the cyclic AMP step suggesting a requirement for synthesis of a specific protein or increased levels of certain proteins for the cyclic AMP effect (Eskin, Yeung & Klass, 1984; Yeung & Eskin, 1987).

Comparison of our results in rat SCN with the sensitive circadian periods in the *Aplysia* ocular pacemaker reveal a surprising correlation. The sensitive period of rat SCN to phase advance by serotonin is CT 3–9; that of *Aplysia* eye is CT 5–11. This temporal correlation of circadian pacemaker sensitivity to serotonin- and cyclic AMP-induced phase shifts in such phylogenetically distant organisms suggests conservation of circadian pacemaker mechanisms. The maximum phase shifts observed in the rat ($7{\cdot}0 \pm 0{\cdot}1$ h) were significantly greater than the 3·5 h phase shift in *Aplysia* (Eskin *et al.* 1982). The treatment times that we used were also more than an order of a magnitude shorter, 5 min *vs.* 1·5 h. The mechanism underlying these differences in sensitivity is unclear, but such differences are characteristic of the respective sensitivities of these two organisms to other circadian phase-shifting stimuli.

The phase shifts observed after treatment with serotonergic agonists suggest that a 5-HT_1 receptor may be involved in mediating serotonergic signals to the SCN. Neither definitive classification, nor functional and anatomical distribution of the serotonergic receptor subtypes present in the SCN has been determined. The 5-HT_1 receptors comprise a family of receptor subtypes that are radiolabelled with [^{3}H]5-HT. The 5-HT_{1A} receptors have been identified by radiolabelling with [^{3}H]8-OH DPAT (Middlemiss & Fozard, 1983; De Vivo & Maayani, 1986). They are a distinct

group of receptors that work through an adenylate cyclase pathway in a number of systems. There is conflicting evidence in terms of the mechanism of action of the 5-HT_{1A} receptors, as there are some reports that indicate that it acts through elevation of cyclic AMP levels (Markstein, Hoyer & Engel, 1986), while others suggest that it is negatively coupled to cyclic AMP (Weiss, Sebben, Kemp & Bockaert, 1986). Our results not only confirm the specificity of the serotonin-induced phase shifts but also lend support to the notion of a possible cyclic AMP-coupled mechanism.

The effects of quipazine, a non-specific serotonergic agent with reported agonistic and antagonistic effects (Peroutka, 1988), have been tested with bath application to SCN-containing hypothalamic slices (Prosser, Miller & Heller, 1990). Quipazine induced more modest (4 h) phase advances of SCN rhythms in the day-time compared with serotonin; treatment at night induced 4 h phase delays. That study differed both pharmacologically and methodologically from ours in ways that would contribute to the differing results. It is surprising that the hour-long exposure of the whole slice to a higher concentration of quipazine should produce significantly lower amplitude phase advances than a very localized, brief application of a lower concentration of serotonin. However, an extensive range of serotonin receptor sites and subtypes have been reported throughout the hypothalamic region included in the slice (Dean, Miller & Dement, 1990) and all would be exposed to quipazine during bath application.

Together these two studies suggest that there may be topographic variations in the function of serotonin receptors in the SCN and nearby hypothalamus. In the day-time quipazine may have produced different effects at the various serotonin receptor sites and types that summed to damp the full shift inducible at dorsal raphe projection sites. More intriguing is the phase delay produced at night by quipazine, but not serotonin or 5-carboxamidotryptamine. It cannot be explained by instability of the serotonin solution: serotonin was applied within 15 min of preparation and 5-carboxamidotryptamine, a stable analogue, produced the same responses. The finding that phase delays were stimulated only with bath application suggests that perhaps phase delays and phase advances are mediated through different pathways in the mammalian circadian system. Serotonergic pathways that terminate at hypothalamic regions near the SCN, but do not overlap with the retino-hypothalamic, geniculo-hypothalamic or raphe projections to the ventrolateral SCN would be candidates. This provocative hypothesis deserves further investigation.

Our study demonstrates a regulatory role for serotonin in the rat SCN. While the raphe input is not necessary to sustain circadian rhythmicity *in vitro*, it may play an integrative role in the temporal organization of the mammalian circadian system. The raphe acts to modulate arousal states and integrate behaviour in response to changes in motor activity, wakefulness and other physiological functions. In the circadian system serotonin from the raphe may serve as a messenger of daily, rhythmic changes in the arousal state of the animal. Stimulation of the serotonergic system alters the phase of the pacemaker *in vitro*, and it may function similarly *in vivo* to synchronize the pacemaker to social or behavioural changes in the environment.

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~~ELECTROPHYSIOLOGY OF THE SCN CLOCK:~~ in the suprachiasmatic nuclei
INTRINSIC NEURONAL RHYTHMS AND THEIR ADJUSTMENT

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Left: Gillette et al
Running Title: ~~SCN~~ Neuronal Rhythms in the SCN ~~and their Adjustment~~

Abstract. The central role of the suprachiasmatic nuclei (SCN) in regulating mammalian circadian rhythms is well established. We study the temporal organization of SCN neuronal properties in the SCN using a hypothalamic brain slice preparation from rat. Electrical properties of single neurons are monitored by extracellular and whole cell patch recording techniques. The ensemble of SCN neurons in the SCN undergoes circadian changes in spontaneous activity, membrane properties and sensitivity to phase adjustment. At any point in this cycle, diversity is observed in individual neurons' electrical properties, including firing rate, pattern and response to injected current. Nevertheless, SCN generate stable, near 24 hr oscillations in ensemble neuronal firing rate for at least three days *in vitro*. The rhythm is sinusoidal, with peak activity, a marker of phase, appearing near midday. In addition to these electrophysiological changes, the SCN undergo sequential changes *in vitro* in sensitivities to adjustment. During subjective day, the SCN progress through periods of sensitivity to cyclic AMP, serotonin, neuropeptide Y, and then to melatonin at dusk. During the subjective night, sensitivities to glutamate, cyclic GMP and then neuropeptide Y_X are followed by a second period of sensitivity to melatonin at dawn. Because the SCN, when maintained *in vitro*, are in constant conditions and are isolated from afferents, these changes must be generated within the SCN in the SCN clock. The changing sensitivities reflect underlying temporal domains that are characterized by specific sets of biochemical and molecular relationships which occur in an ordered sequence over the circadian cycle.

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← The paired suprachiasmatic nuclei (SCN) of the hypothalamus are the seat of the primary circadian time-keeping mechanism of mammals. The SCN mark the passage of time in near 24-~~hr~~ hour cycles. It is here that entraining signals, initiated by environmental change, integrate and ~~act~~ are so as to adjust the phase of the pacemaker. Efferent signals from the SCN organize and regulate metabolic, physiological and behavioural functions that occur in circadian patterns. ~~While a number of the papers~~ Other chapters in this ~~symposium~~ volume address aspects of the mammalian circadian system studied in whole organisms, ~~studies, this paper will~~ whereas we shall examine ~~SCN~~ the neuronal and pacemaker properties of the SCN that are expressed in a hypothalamic brain slice. Because our studies evaluate SCN isolated *in vitro*, we directly assess intrinsic properties. We have found that the SCN ~~is~~ are composed of an electrophysiologically diverse group of neurones that function remarkably autonomously: The SCN generate stable, near 24-~~hr~~ hour rhythms of ensemble neuronal activity *in vitro* and undergo an orderly sequence of changes in sensitivity to stimuli that ~~induce~~ adjust phase ~~adjustment~~. The pattern of responsiveness to neurochemicals known to be contained in SCN afferents demonstrates that the organization of the clock ~~organization~~ is more complex than simply night ~~vs~~ -versus- day processes; rather, our findings suggest there is a continuously changing series of sensitivities to multiple phase-adjusting stimuli. This sequence must reflect changes in underlying cellular processes, or *temporal domains*. These domains are functional epochs, characterized by specific biochemical or molecular substrates and their interactions, which are linked together to generate the daily order of the circadian clock's cycle.

The experimental system that we ~~study~~ use is ~~the SCN brain~~ a hypothalamic slice from inbred Long-Evans rats ~~of~~ 5-10 ~~wk~~ weeks old. It differs from ~~organismic~~ whole organism studies of clock function in several important ways. First, we have dissected the SCN out of the central nervous system into a 500 μm coronal slice of hypothalamus. Therefore, the activities and circadian properties that we measure must be generated spontaneously within the tissue slice. The slice contains less than the 800 μm ~~rostral-caudal~~ extent of the nucleus; we study properties of the medial portion, primarily. ~~It follows that~~ any circadian patterns that we observe ~~would be~~ therefore are the result of activity in less than the entire nucleus and would indicate that there is redundancy in pacemaker organization.

Second, the suprachiasmatic slice is studied in a defined, constant environment with minimal supplementation. The fresh slice is placed in a brain slice chamber at the interface of a moist, 95% O_2:5% CO_2 atmosphere and the glucose-bicarbonate-supplemented medium (Earle's Balanced Salt Solution, 37 °C), maintained at pH 7.4 and perfused at 34 ml/h (see Prosser ~~and~~ & Gillette, 1989 for complete methods). The SCN are clearly visible in the slice, which gives ~~This permits~~ the investigator ~~to have~~ precise control over the sites of measurement and drug treatment. The *in vitro* preparation thus has the advantage of a high degree of control over the chemical and physical environment while manipulations are made under clear visual guidance.

Third, the SCN that we study within the brain slice are removed from the influence of other brain regions and humoral factors. After the suprachiasmatic slice has been cut, the base of the hypothalamus is surgically reduced to exclude the supraoptic nuclei,

although part of the paraventricular nuclei may be included in the slice because the third ventricle is intact dorsally. Thus, in the present studies, the SCN are free of the primary brain regions that project to and regulate them, as well as most feedback loops. Electrical properties, spontaneous activities and sensitivities to chemical perturbations are assessed directly in the free-running clock with little, if any, contamination from signals from other sites. Our measure of phase is the peak of the circadian rhythm of firing activity of the neuronal ensemble, which most probably represents both intrinsic signalling and the primary neural output of the clock. Any circadian changes that we measure *in vitro* are those generated spontaneously within the SCN, that is, those which are components of or are driven by the timekeeping mechanism.

A primary property attributed to the SCN from organismic studies is that of endogenous oscillation with a period about that of the day, *i.e.*, circadian. This behaviour can be studied in our brain slices because maintained under these conditions we use as they survive for at least three days *in vitro*. At any one time point within the daily cycle, SCN neurones show diversity in rates and patterns of spontaneous activity. Circadian measurements made with whole cell patch recording methods in the brain slice (Blanton *et al.*, 1989) demonstrate that, electrophysiologically, the SCN may be composed of considerably more cells types than first reported (Wheal and Thompson, 1984). Preliminary determinations found that 75% of over 50 neurones sampled were spontaneously active, with firing patterns ranging from very regular to irregular random and burst patterns (Gallman and Gillette, 1992). While neurones of the various types could be found across the circadian cycle, but circadian patterns of firing frequency,

membrane potential and specific membrane conductance were apparent (Gallman and Gillette, 1993). Nevertheless, circadian changes in firing frequency of the sample measured with whole cell patch recording resemble the sinusoidal oscillation recorded extracellularly for the neuronal ensemble (Fig. 1, Gillette, 1991): Mean firing rate peaks mid-subjective day, alternating with a trough during subjective night.

The pattern of oscillation in spontaneous activity of the ensemble of single units monitored extracellularly is remarkably stable over multiple 24-hr cycles, so that the time of peak activity is a reliable marker of phase (Prosser and Gillette, 1989). Despite the fact that the tissue contains significantly less than the entire SCN and is maintained in glucose-supplemented mininal salt solution, the period of the daily oscillation is near 24-hr. These observations reveal that isolated SCN are able to spontaneously regenerate circadian rhythmic neuronal activity cycles with only an exogenous energy source. Further, because this occurs regularly in coronal slices which inevitably cut through some portion of the SCN, the pacemaker must be redundant within the rostral-caudal SCN (Gillette, 1991).

A second property attributed to circadian clocks from study of behaving organisms is differential sensitivity to phase adjustment. This was tested in SCN *in vitro* with structural analogs of the ubiquitous second messenger (cAMP). A number of 8-substituted cAMP analogs, including 8-benzylamino-cAMP (BA-cAMP), 8-bromo-cAMP (Br-cAMP) and 8-chlorophenylthio cAMP, when bath applied for ≤1 hr during subjective daytime, permanently advanced the time of peak neuronal activity; non-cyclic

8-BA-5'AMP was ineffective (Gillette and Prosser, 1988; Prosser and Gillette, 1989). When SCN were treated at midday, peak firing appeared 4.5 h before the expected time of peak for the two cycles monitored thereafter. These analogs were ineffective at phase adjustment during subjective night (Fig. 2). Likewise, treatments that elevate endogenous cAMP, such as forskolin or RO 20-1724, were effective only in the daytime. Conversely, Br-cGMP, a structurally related purine cyclic nucleotide analog, was effective in antiphase to Br-cAMP and BA-cAMP; it adjusted SCN phase only at nighttime (Fig. 2, Prosser *et al*, 1989.) These were the first demonstrations of not only phase adjustment in the isolated SCN, but also of spontaneous waxing and waning of one sensitivity followed by another. With these findings persistence of SCN clock properties *in vitro* was established.

Both cAMP and cGMP are potent regulators of cell state. Their 8'-analogs are exceptional activators of the respective protein kinases regulated by these cyclic nucleotides, and are only slowly degraded (Meyer and Miller, 1974). Differential clock sensitivity to each over the circadian cycle may be modulated at multiple levels. While the concentrations of these kinases does not appear to change, the levels of their regulatory cyclic nucleotides as well as the phosphorylation states of the kinases and some substrates oscillate over the 24-h cycle (Prosser and Gillette, 1991; Faiman and Gillette, 1992; Weber and Gillette, 1992). The critical point at which these changes adjust the clock mechanism has yet to be determined.

The identity of synaptic neuromodulators mediating phase adjustment, including

those activating these second messengers, is not yet known; however, ~~a number of~~ candidates ~~candidates~~ are ~~suggested~~ known to be neuroactive substances localized in afferent fibers and by those with ligand binding sites within the SCN. Major projections to the SCN include those from: (1) the dorsal raphe, containing serotonin (5-hydroxytryptamine, 5HT); (2) the intergeniculate leaflet (IGL) of the lateral geniculate nucleus, containing neuropeptide Y (NPY) and GABA; and (3) the retina, via the retinohypothalamic tract (RHT) fibers containing a glutamate ~~(GLU)~~ precursor (Fig. 3). Additionally, the SCN is one of the few brain regions that bind significant amounts of melatonin, ~~(MEL)~~, the indoleamine produced nocturnally by the pineal. We have explored the sensitivities of the SCN to candidate modulators.

Neurones of the ventrolateral SCN (VLSCN) of the rat receive serotonergic fibers from the dorsal raphe (Azmitia ~~and~~ & Segal, 1979, Moore *et al*, 1978, van de Kar ~~and~~ & Lorens, 1979), as well as NPY- and ~~GLU~~ glutamate-containing terminals from the IGL and retina, respectively (Card ~~and~~ & Moore, 1988, Mikkelsen ~~and~~ & O'Hare, 1991, Castel *et al*, 1993). This indicates that the VLSCN in the rat is a major site of signal integration; furthermore, because these fiber types may synapse both upon each other and on the same SCN neurone (van den Pol ~~and~~ & Gorcs, 1986, Guy *et al*, 1987), this integration may have both pre- and post-synaptic components. Cognizant of the localized nature of the termination sites of these projections, ~~termination sites~~, we designed these experiments to examine the effects of localized application of test substances in microdrops applied directly to VLSCN.

Both 5HT and NYP adjust SCN phasing in the SCN when applied *in vitro* during subjective

daytime. The period of sensitivities to these two neuromodulators encompasses the period of phase-advance induced by non-photic stimuli (Mrosovsky *et al.*, 1989; and see Mrosovsky, 1994 this volume.) When 10^{-6} M 5HT was applied to the VL-SCN in a 10^{-11} ml droplet for 5 min, significant phase adjustments (>1 hr) were observed between CT 2-10 (Fig. 4; Medanic and Gillette, 1992). The maximal response occurred at CT 7 when 5HT treatment induced a 7.0±0.1 hr phase advance. This period of sensitivity to 5HT microdrop overlaps with but is not identical to that for bath-applied cAMP (Fig. 4). While differences in the details of 5HT and cAMP phase-response curves (PRCs) could reflect integrative responses due to the differing extent of exposure to the stimulus between microdrop and bath application, the similarities, especially between CT 2-5 are striking, and suggest a common pathway. Agonist and antagonist studies have implicated a $5HT_{1A}$-like receptor (Medanic and Gillette, 1992, Shibata *et al.*, 1992, Prosser *et al.*, 1993). Indeed, a new cAMP-coupled 5HT receptor ($5HT_7$) has recently been cloned from rat brain (Lovenberg *et al.*, 1993); Although it has not been localized to the SCN, should this receptor reside either in VL-SCN neurones or in presynaptic terminals, it could modulate the early phase of the 5HT shift by a cAMP-dependent mechanism. The disparity between the cAMP and 5HT phase response curves after this point would suggest that the latter response, from CT 6-10 and including the maximal effect, is mediated at least partially by a non-cAMP-dependent mechanism.

Fig. 4

Daytime sensitivity to NPY microdrops applied to VL-SCN significantly lags the sensitivities to cAMP and 5HT (Fig. 4; Medanic and Gillette, 1993). It first appears at CT 5, peaks at CT 8 with a maximal phase shift of 4.5 hr, about 2.5 hr less than the 5HT

peak and then dissipates in parallel with the waning 5HT response. This temporal pattern of SCN sensitivity to NPY in subjective day makes it unlikely that NPY utilizes a cAMP-stimulating signal transduction mechanism. A second period of sensitivity to NPY anticipates dawn, peaking at CT 22, a time when SCN are insensitive both to cAMP and cGMP regulation. This bimodal pattern of sensitivity of the free-running SCN to NPY raises questions regarding the mechanism(s) regulating sensitivity. Is the receptor itself disappearing, then reappearing, or is regulation at the levels of signal transduction elements, or at cellular substrates of the transduction cascade? Our results regarding changing sensitivity to cAMP stimulation would suggest that the latter relation is most likely. What ever the level(s) of regulation, these changes must be close to or driven by the clock, and thus understanding them should lead us toward a better understanding of time keeping elements.

Nocturnal sensitivity of the SCN to phase adjustment must include the neural substrates of photic entrainment. Because multiple lines of evidence support a role for glutamate (GLU) in mediating signals of light in the environment at RHT synapses at the VL SCN (Rusak and Bina 1990; Kim and Dudek 1992), we examined temporal changes in the effect of focal application of GLU to the SCN. One μl drops, which effectively covered one SCN with 10 mM GLU for 10 min, produced phase delays and phase advances which are remarkably light like in time, shape and amplitude. Maximal delay at CT 14 and maximal advance at CT 18 (Ding and Gillette 1993). Interestingly, while this period of sensitivity spans that to cGMP, the bimodal shape and lesser amplitude of the GLU response suggest that activation of cGMP pathways cannot

wholly explain the ~~GLU~~ result with glutamate.

Interspersed between the daytime and nighttime sequences of ~~SCN~~ sensitivities of the SCN to these neuromodulators are periods that represent the entrained light-dark and dark-light transition zones. The first of these, which surrounds environmental dusk, coincides with a period when injections of the pineal hormone melatonin ~~(MEL)~~ have been shown to entrain rats and alter energy utilization in the SCN (Redman *et al.* 1983, Cassone *et al.* 1986, 1988). Not surprisingly, bath application of 10^{-9} M ~~MEL~~ melatonin for 60 min adjusts the subsequent cycles of ~~SCN~~ firing rate (in the SCN) (McArthur *et al.* 1991) by advancing the clock up to 4.5 hr. Careful examination of ~~SCN~~ sensitivity surrounding dawn revealed a second, narrow period of sensitivity to ~~MEL~~ melatonin that appeared sharply at CT 23 and then decayed over the next ~~2 hr.~~ two hours (McArthur & Gillette, 1994). Both of these sensitive periods closely follow those to NYP. In this respect, ~~MEL~~ melatonin ~~closely~~ resembles NPY: Both show two windows of sensitivity, separated by many hours, ~~and these~~ which appear spontaneously in the constant conditions *in vitro* without exposure to the phase-adjusting agent. In the case of ~~MEL~~ melatonin, these sensitive periods occur at times when seasonal changes in nightlength can be expected to stretch the duration of pineal ~~MEL~~ melatonin synthesis into these temporal domains of the SCN.

~~Viewing the present data as a whole~~ Overall, we have accumulated evidence that the properties of a circadian clock persist in SCN *in vitro*. The SCN generate multiple, near 24 hr cycles of ensemble neuronal firing rate and show differential sensitivity to phase-adjusting stimuli. The patterns of membrane properties and neuronal activity are circadian even though less than the entire SCN is present in the brain slice. SCN

continue to generate a stable circadian oscillation in the ensemble electrical activity, even though component neurones ~~exhibit~~ show diversity in electrophysiological properties. The contribution of the various electrophysiological types of neurones to integrative or timekeeping functions of the SCN is presently unknown. Further, the isolated SCN ~~proceeds~~ through an orderly sequence of sensitivities to resetting stimuli. This occurs without prior exposure to the stimuli during that circadian cycle. The onset of Each sensitive period is generally ~~shows~~ more rapid ~~onset~~ than its decay ~~offset~~. ~~Each~~ The neuromodulatory stimulus identified ~~thus~~ so far ~~acts~~ through a different signal transduction pathway, which ~~is~~ are insensitive to direct activation during non-sensitive periods. This suggests that the processes that regulate sensitivity operate beyond the level of the receptor, at least one ~~to several~~ steps into the cell. The cellular substrates that determine the sensitivity and ~~reponse~~ the characteristics of the response to extracellular regulators define separate temporal domains which appear as an orderly sequence of biochemical and molecular relationships that together make up the circadian cycle. Understanding the components of each domain, its positive and negative regulators and the linkages between successive domains should permit fine control of phase adjustment.

Acknowledgements

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Figure Legends.

Figure 1. The circadian oscillation in spontaneous firing rate of the ensemble of SCN neurons in a hypothalamic brain slice. Mean firing rate (Hz) for all units encountered with an extracellular electrode and sampled within a 2-hr interval is plotted against circadian time (CT) of the entrained lighting cycle, where CT 0 is time of the dark/light transition in the donor's entraining cycle. The slice was prepared at CT 1 (arrow) from an 8-week-old rat reared in a 12L:12D lighting cycle and placed in constant light in the interface brain slice chamber; recording commenced at CT 4. Activity peaked at CT 7, seven hours into the entrained light cycle, and was generally low at night. Error bars, SEM; n = 6–15 neurons per bin; each 24-hr bin is offset by 15 min to generate a running average.

Figure 2. The rat SCN sensitivity to cAMP is in antiphase to that for cGMP. Plotted are the phase shifts in time of the peak in the electrical activity rhythm in the cycle after treating the SCN *in vitro* for 1 hr with bath-applied analogs. Phase response relationships for analogs (8BA- cAMP and 8Br-cGMP; 8Br-5'AMP was without effect. Sensitivity to stimulating cAMP pathways is confined to subjective day, and to stimulating cGMP pathways to night. (Adapted from Prosser *et al*, 1989.)

Figure 3. Schematic of the head of a rat with major brain sites that regulate the SCN. The SCN is positioned at the base of the hypothalamus, directly over the optic chiasm. It receives 5HT projections from the dorsal raphe, NPY/GABA projections from the intergeniculate leaflet (IGL), and GLU-liberating axons from the retina via the (RHT). Additionally, the pineal (PIN) is the source of circulating MEL.

Figure 4. The family of phase-response curves relating three daytime phase-shifting agents, (BA-cAMP, 5HT or NPY. BA-cAMP was bath applied for 1 hr at 5 X10⁻⁵ M whereas 5HT and NPY were applied at 10⁻⁶M in microdrops to the VLSCN for 5 min.

Notice the similarities and differences amongst this family of curves. The numbers by the points on the 5-HT and NPY curves indicate the number of experiments performed at each circadian time studied. Error bars ± S.E.M. (some are less than the size of the symbol.) Rats were entrained as in Fig. 1. Data replotted from Prosser & Gillette, 1989; Medanic & Gillette, 1992; and Medanic & Gillette, 1993.)

Curves were fit by eye to the following data: BA-cAMP = 25 experiments at 16 CTs from Prosser and Gillette 1989; serotonin = 20 experiments at 7 CTs from Medanic and Gillette, 1992; NPY = 30 experiments at 11 CTs from Medanic and Gillette, 1993. Rats were entrained as in Fig. 1; subjective night is represented by the shaded horizontal bar.

Fig. 1

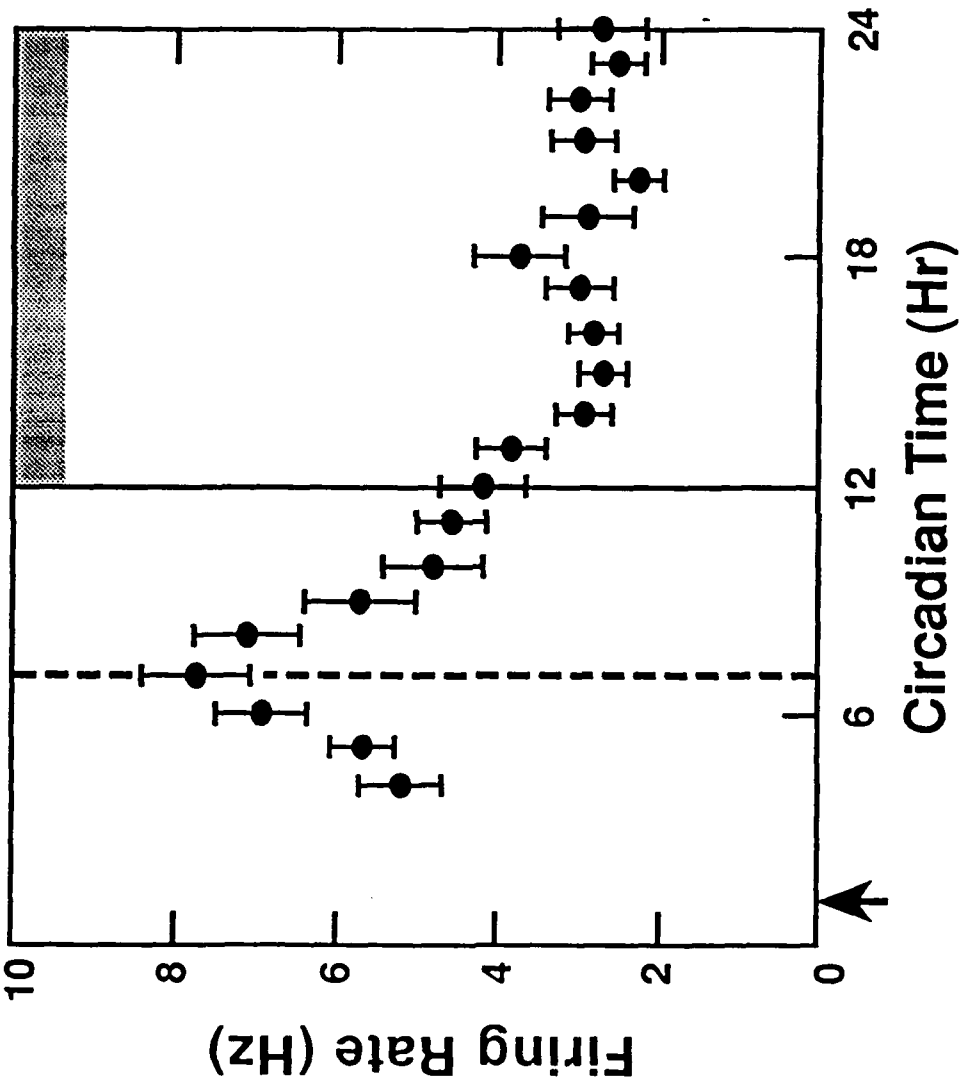

Fig. 2

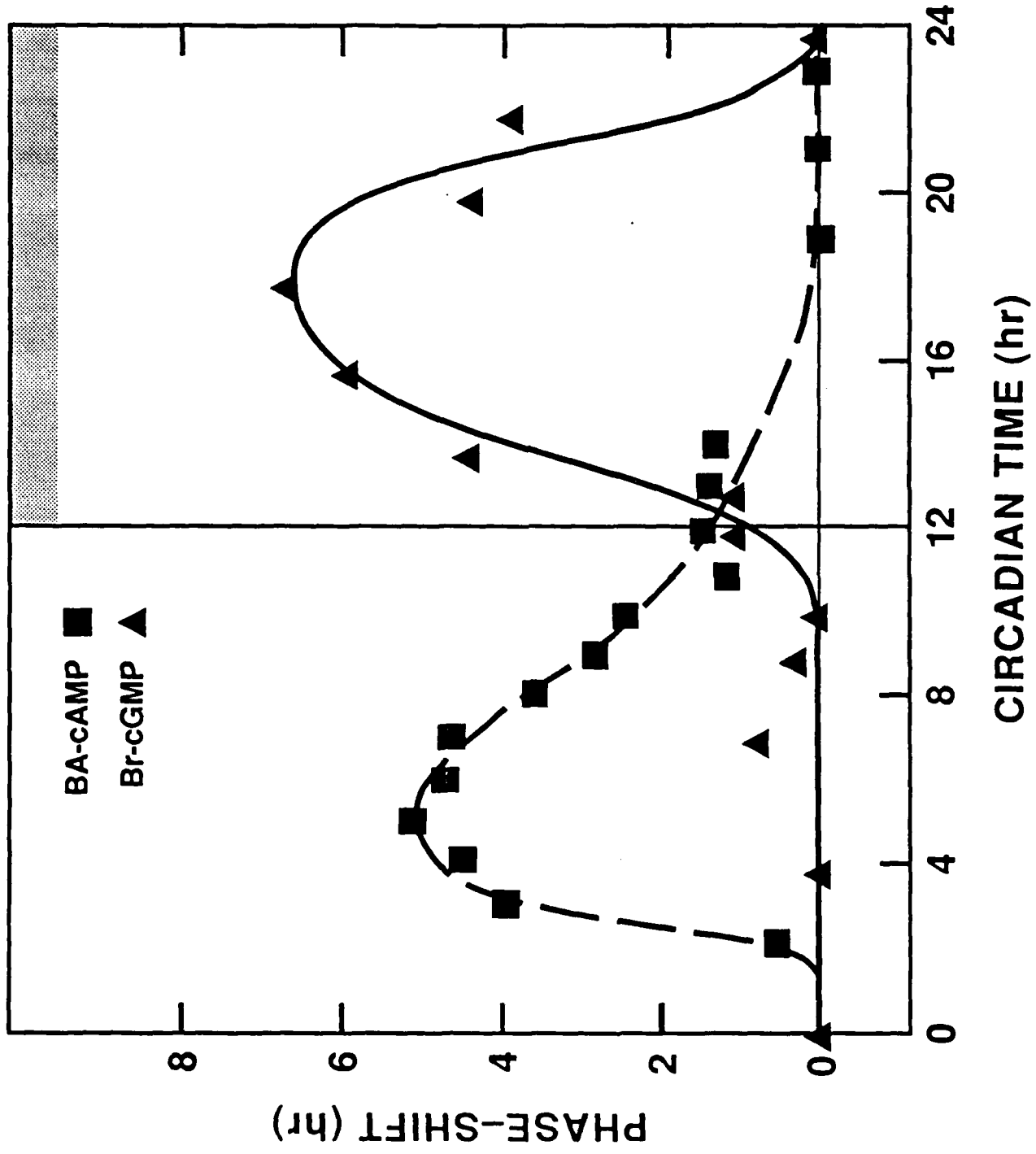
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Fig. 3

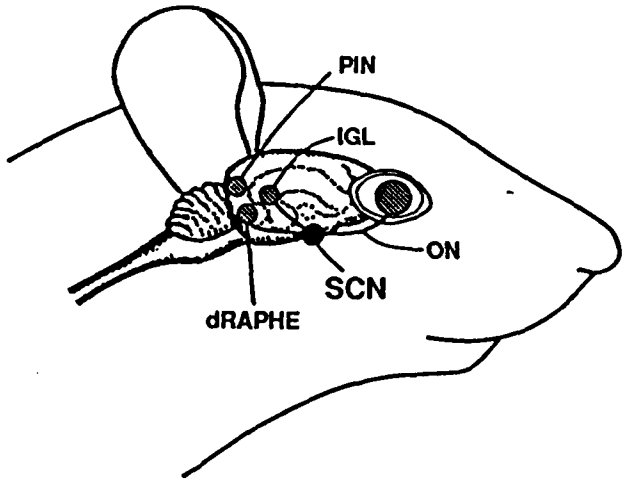
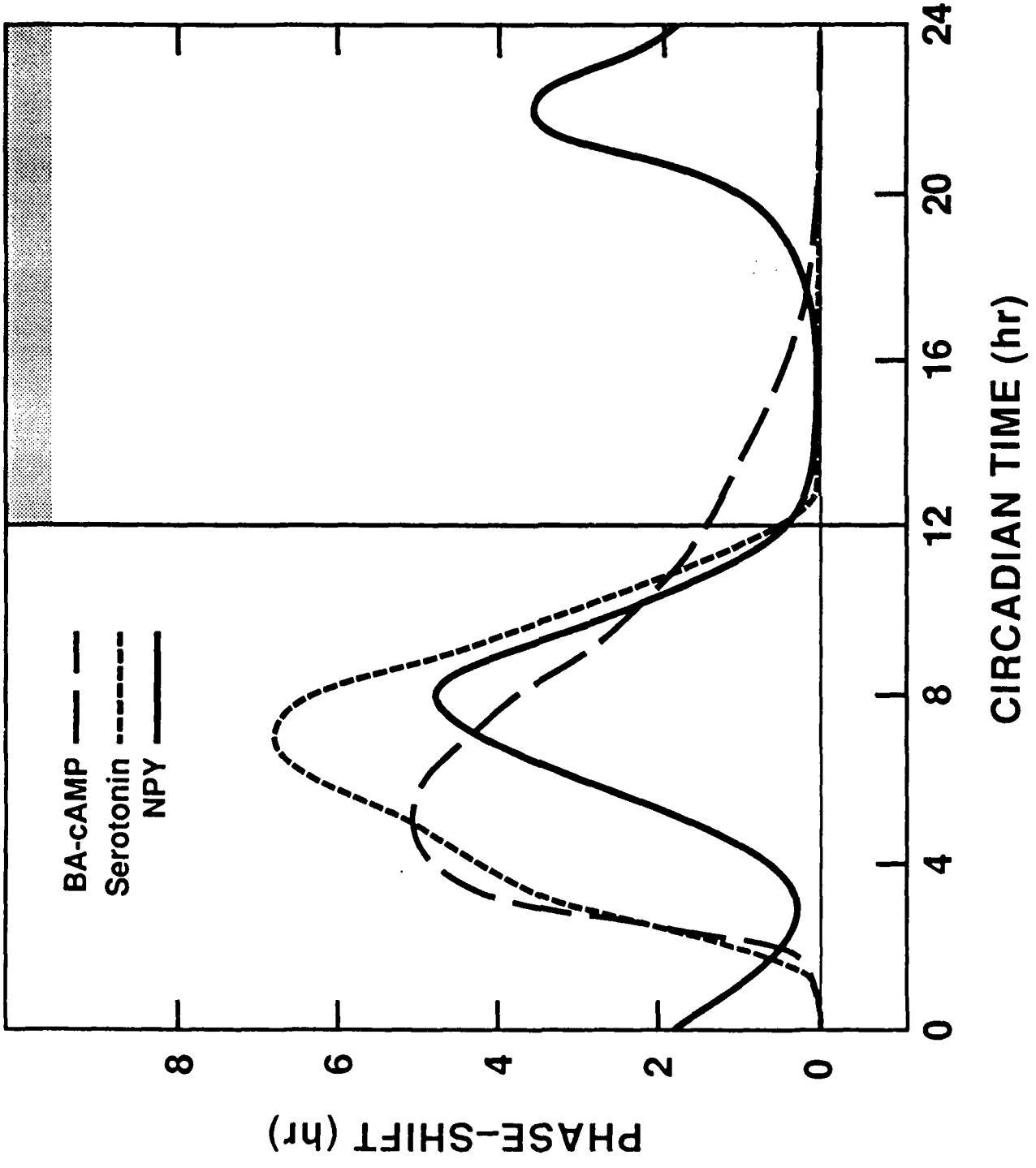


Fig. 4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rapid communication

Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones?

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Satinoff, Evelyn, Hua Li, Thomas K. Tcheng, Chen Liu, Angela J. McArthur, Marija Medanic, and Martha U. Gillette. Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R1216–R1222, 1993.—The basis of the decline in circadian rhythms with aging was addressed by comparing the patterns of three behavioral rhythms in young and old rats with the in vitro rhythm of neuronal activity in the suprachiasmatic nuclei (SCN), the primary circadian pacemaker. In some old rats, rhythms of body temperature, drinking, and activity retained significant 24-h periodicities in entraining light-dark cycles; in others, one or two of the rhythms became aperiodic. When these rats were 23–27.5 mo old they were killed, and single-unit firing rates in SCN brain slices were recorded continuously for 30 h. There was significant damping of mean peak neuronal firing rates in old rats compared with young. SCN neuronal activities were analyzed with reference to previous entrained behavioral rhythm patterns of individual rats as well. Neuronal activity from rats with prior aperiodic behavioral rhythms was erratic, as expected. Neuronal activity from rats that were still maintaining significant 24-h behavioral rhythmicity at the time they were killed was erratic in most cases but normally rhythmic in others. Thus there was no more congruence between the behavioral rhythms and the brain slice rhythms than there was among the behavioral rhythms alone. These results, the first to demonstrate aberrant SCN firing patterns and a decrease in amplitude in old rats, imply that aging could either disrupt coupling between SCN pacemaker cells or their output, or cause deterioration of the pacemaking properties of SCN cells.

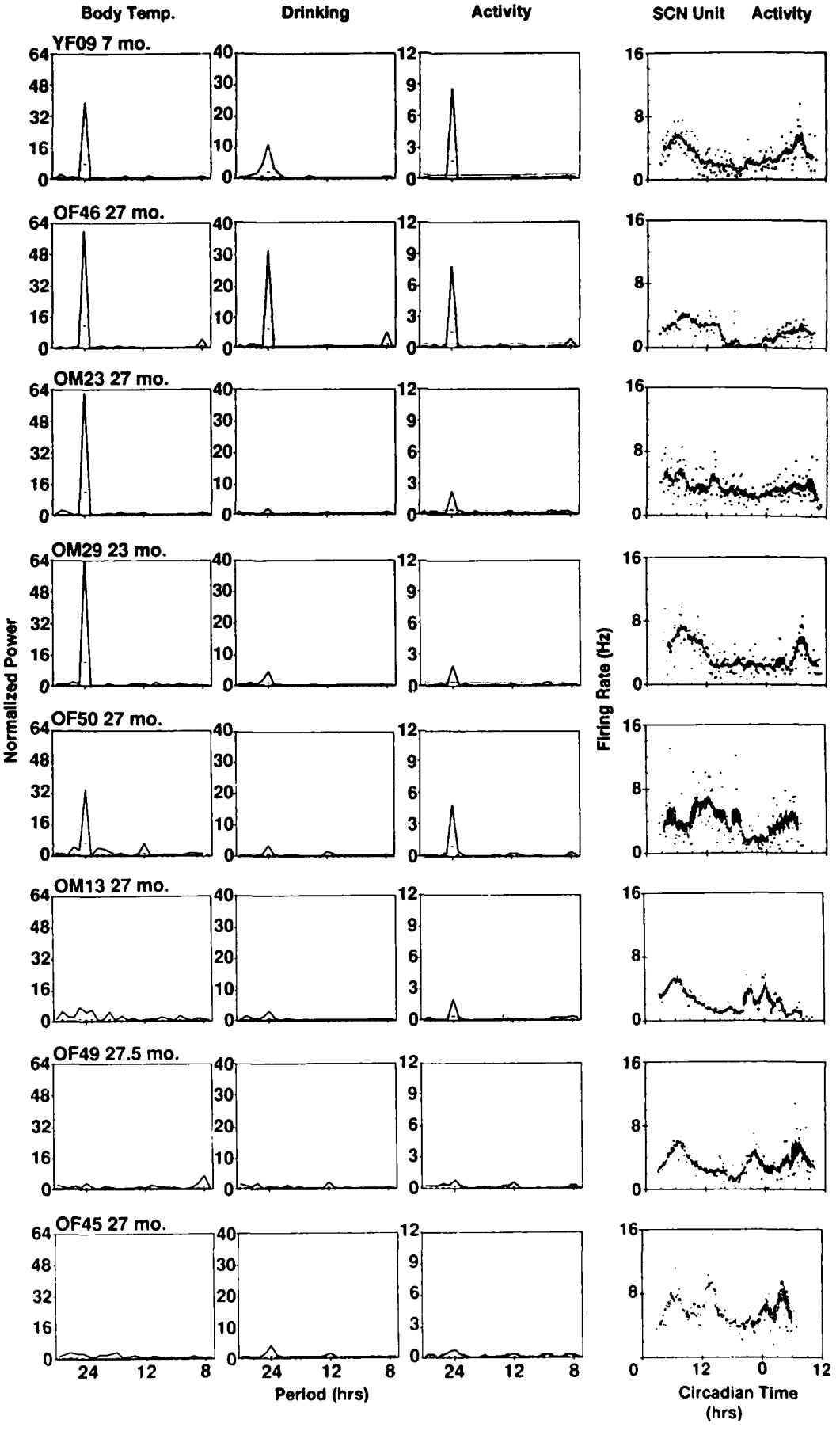
brain slice; circadian rhythms; body temperature; activity; drinking; rat

THE BIOLOGICAL CLOCK in the suprachiasmatic nuclei (SCN) produces timing signals that generate circadian rhythms in physiological and behavioral systems. Normal biological rhythm functioning contributes to good health and well-being (1, 2). During aging, many characteristics of rhythmic behaviors deteriorate; the two most commonly seen changes are decreased amplitude and a disruption of normal patterning (13). There are important questions to be asked about these changes, including possible changing relationships among behavioral rhythms and between the SCN and behavioral rhythms, as well as changes within the SCN. Do all circadian rhythms become disrupted at the same time, or does one invariably deteriorate before another, or is there no pattern to the loss of rhythmicity? Does the deterioration reflect changes in the circadian clock itself or in the interaction between the clock and the overt behaviors it regulates?

Changes in rhythmic behaviors could be due to alterations in rhythmic neuronal activity in the SCN of the hypothalamus, a principal pacemaker that organizes circadian rhythms in mammals (18). In rats, SCN lesions disrupt circadian rhythmicity in many behaviors (see Refs. 15 and 19 for review). The SCN generate a near 24-h rhythm of multiunit activity in vivo (8). When isolated in vitro, the SCN continue to produce a circadian rhythm of electrical activity (5, 6) that is stable for multiple cycles and matches the rhythm in vivo (11). The rhythm of 2-deoxyglucose uptake in an SCN slice is proportional to the rate of glucose utilization in light-entrained rats (10). Cultured SCN cells retain the capacity for circadian oscillation (9). Thus the SCN in vitro is an appropriate preparation for assessing SCN rhythms.

Homeostatic regulations also deteriorate in aging organisms (7, 12). This leads to the question of whether the decline in circadian rhythmicity might be a masking effect. Masking usually refers to an environmental cycle exerting a strong exogenous influence on a biological variable without a direct effect on the underlying timing process. We use the term here to refer to a physiological system exerting a strong effect on its overt rhythm. For instance, if renal insufficiency or vasopressin dysfunction caused increased drinking during the day, the drinking rhythm might disappear, but this would not imply loss of control of the rhythm via the pacemaker. Similarly, if a decrease in one rhythm (e.g., activity) caused a reduction in another rhythm to which it is linked (e.g., body temperature), this would also be a masking effect.

Although we did not look for masking effects, we did try to determine whether the deterioration of circadian rhythms in elderly rats was accompanied by changes in rhythmic neuronal activity in the SCN. If changes in behavioral rhythmicity were mirrored by changes in this measure of output of the primary pacemaker, then we could infer that the changes in the behaviors were caused by alterations in neuronal relationships very close to the oscillator as well as possible deteriorations in homeostatic regulations further downstream.


Body Temp.
Drinking
Activity
SCN Unit Activity
YF09 7 mo.
OF46 27 mo.
OM23 27 mo.
OM29 23 mo.
OF50 27 mo.
OM13 27 mo.
OF49 27.5 mo.
OF45 27 mo.
Normalized Power
Firing Rate (Hz)
Period (hrs)
Circadian Time (hrs)

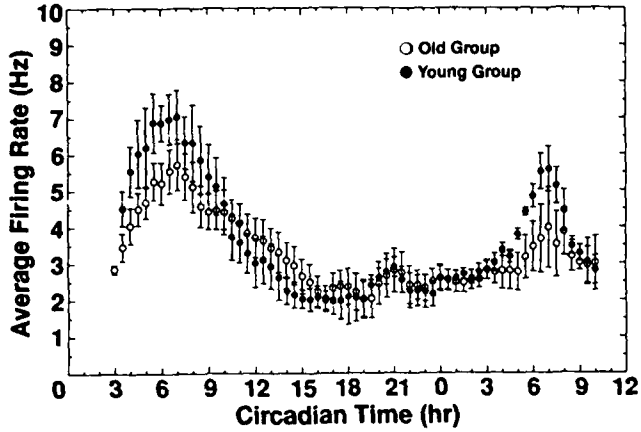


Fig. 3. Circadian variation in the ensemble firing rates within the old (n = 8) and young (n = 4) groups of rats. Two-hour sliding window averages of neuronal firing rates were calculated for individual subjects at 30-min intervals. These mean values were averaged within each group to produce the plotted values. Group averages for time points with fewer than two subjects were not calculated. The amplitude is lower for the old group (P = 0.016, see text).

by visual inspection of a graph of these values for the symmetrically highest point. Recording was performed blind, i.e., without knowledge of the previous behavioral history or age of the rats. In addition to the 13 rats whose behavioral histories had been recorded, slice recordings were also made from two 2-mo-old rats on which prior behavioral rhythms were not recorded.

Curve fitting and statistical analyses. For curve-fitting analysis, single-unit raw data points were grouped into overlapping 2-h bins at 30-min intervals. Such sliding window averaging acts as a low-pass smoothing filter that reveals long-term temporal changes by smoothing their inherent heterogeneity. A periodic parametric curve was fitted to each subject's sliding window averages. The form of the curve was designed to describe the typical shape of a young rat's sliding window average. The equation of the curve is Hz = offset + exp[(amplitude * sin (phase)], with phase = [(CT1)/24] * 2π. Offset and amplitude are parameters that are optimized. Phase fixes the time of peak for the curve at CT7.0, the normal time of peak for young rats (11). Parameter values for the old and young groups were compared using t tests.

RESULTS

Body temperature, activity, and drinking rhythms were recorded for 35–178 days. Figure 1 shows these patterns of body temperature, activity, and drinking of one young and seven old rats for the last 1–2 mo before death. It is readily apparent that there is no consistency between age and rate of decline in circadian rhythms in these outbred rats. One extreme was rat OF46 in which all three rhythms remained strongly entrained to the LD cycle, as was true of the young rats whose SCN activity patterns were analyzed in vitro and 30 other young rats in the laboratory. The other extreme is represented by rat OF45, which had no body temperature or drinking rhythm but maintained a weakly significant activity rhythm. Two male rats, OM23 and OM29, had very similar, strongly entrained patterns of body temperature and weaker rhythms of activity and drinking. The body temperature rhythm in a third male, OM13, appeared to free-run while activity and drinking rhythms were still discernible.

Periodograms for the last 10 days of data for the rats in Fig. 1 are shown in Fig. 2. In general, the behaviors of the old rats were very variable with respect to rhythmicity. One behavior could be rhythmic without any correlation between it and the presence or strength of rhythmicity in the other two behaviors. In four of the old rats body temperature showed a strong 24-h period, while at the same time, the 24-h periodicity of activity and drinking might also be strong (OF46), be considerably weakened (OM23, OM29), or show more power in one rhythm (drinking) than another (activity; OF50). A fifth rat (OM13) had many weakly significant ultradian frequencies in body temperature and weak circadian drinking and activity rhythms. The body temperature rhythm of OF49 had a weak 8-h periodicity. At the same time there was a weak 12-h and 24-h periodicity in the drinking rhythm and a 12-h rhythm in activity. OF45 lost its body temperature and drinking rhythms but maintained a circadian period in activity. In summary, these three behavioral output measures of the pacemaker were not correlated with each other either within an individual rat or between rats.

The right side of Fig. 2 shows the 30-h free-running pattern of the ensemble single-unit activity in the SCN slice; recording began 1 h after the rats were killed. The top record (YF09) is characteristic of SCN activity in young rats (11), with a sinusoidal pattern that has a single peak in SCN unit activity at CT7 on both *day 1* and *day 2* and a peak amplitude of ~6 Hz. This agrees closely with previous work with respect to time and amplitude of the first peak recorded in vitro (11). The brain slice recording of OM29 looks like that of a normal young rat, with a single peak per day at around CT7. Yet the SCN unit activity of OM23, whose behavioral rhythms are essentially identical to those of OM29, was the flattest of any of the old animals, with no clear initial peak. OF46, which had three strong behavioral rhythms, had a slice rhythm that did not have a sharply defined peak. The other four rats shown in Fig. 2 had aberrant patterns of SCN neuronal activity. Most rats had multiple peaks that appeared at unexpected times, and they were not the same time for any rat (e.g., OF50, at CT4, 10, 12, and 18, OM13 at CT21 and 24, OF49 at CT22, and OF45 at CT13–14, 0–1, and 4). Thus there was no more correlation between this fourth measure of pacemaker output, pattern of SCN firing rate, and the three behavioral measures, than there was among the behavioral measures themselves.

Fig. 2. *Three left columns*: periodograms of body temperature, activity, and drinking of the same rats as in Fig. 1 for the 10 days before the slice recording. The periodogram was normalized by making the total variance = 1. The power of each variable can be compared with the power for the same variable in other rats but not with different variables in the same rat. The horizontal line is the white noise level. The dashed line (in some cases only visible in the graphs with high power) is the lower limit of the 99.99% confidence interval for each frequency. *Right column*: SCN unit activity recorded in constant light for at least 30 hr. Solid line is 2-h sliding window average with data grouped in 15-min intervals. Circles are individual cells.

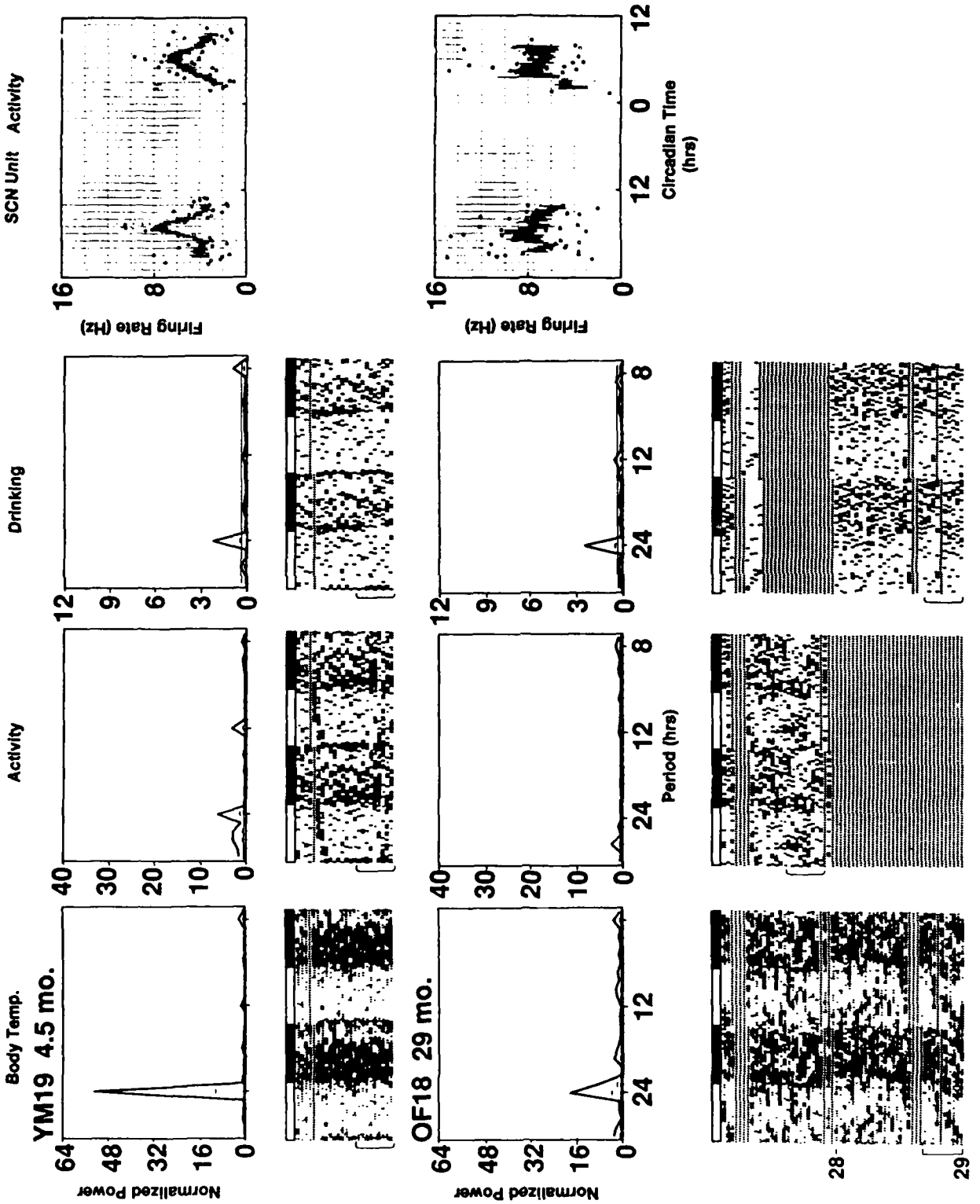


Fig. 4. Three left columns: periodograms of body temperature, activity, and drinking of young male rat YM19, 4.5 mo-old and old female rat OF18, 29-mo-old. Right column: SCN unit activity recorded in constant light for 7 h, from CT3–10 on *day 1*, and at the same time on *day 2*.

Because preparing the brain slice releases each SCN into constant environmental conditions in vitro, an analysis comparing mean SCN firing rates between old and young rats addresses the endogenous rhythmic properties of the old vs. young SCN (Fig. 3). Parameter values for the old and young groups were compared by *t* test. The *t* value for the offset term was not significant ($P = 0.737$), suggesting that overall firing rates were not significantly different from each other. This implies that the high firing cells are not clustered around a particular peak but are more spread out. However, the offset parameter only measures overall firing rates indirectly by adjusting the fitted curve up or down on the Hz axis. A stronger comparison of overall firing rats is a *t* test comparing cell firing rates themselves. Therefore we calculated a mean for each slice and compared old vs. young. There was no significant difference between the groups ($P = 0.322$). This supports the conclusion that the peaks are more scattered in the old group rather than that the high firing cells have disappeared.

The *t* value for the amplitude term was significant ($P = 0.016$). The mean amplitude values were 1.375 for the young rats and 0.876 for the old rats. This difference indicates that the amplitude of the circadian oscillation in SCN neuronal firing rates is attenuated in old rats. This change in amplitude reflects underlying changes in the behavior of individual neuronal activities such that there is apparent loss of coherence of the timing of appearance of fast units, producing multiple peaks (Fig. 2, *right*), as well as a general increase in variance of the activity, both fast and slow, of the neuronal population at any one time. The nature of these differences can be seen by comparing two behavioral rhythms and slice firing rate from a 5 and a 29-mo-old rat (Fig. 4). In these cases the units were recorded for 7 h, the slice was left untouched for 14 h, and then recordings were made again for 7 h to sample only the peak activities. In the young rat, the firing rates on the second day were as high as on the first day. This is in contrast to the old rat: there was an initial drop in firing rate on the first day, which is never seen in slices from young animals (11), and there were no clear midday peaks. Even when firing rate is damped in slices from young rats, a single major peak at CT6–7 is still clearly seen. In any case the firing rates on the second day in some old rats, such as OF45 and OF49, showed no such damping (Fig. 2).

DISCUSSION

There are two major points in this study. The first is that there is a loss of coherence of behavioral rhythms in aged rats. Circadian rhythmicity in body temperature, drinking, and locomotor activity did not deteriorate in the same order in individual elderly rats. This may result from several causes. Although masking effects due to peripheral factors may have contributed to some disruptions in the behavior, they cannot wholly explain the aggregate data. The latter can be accounted for by assuming that pacemaker or output cells in the SCN consist of coupled oscillators. Whenever sufficient numbers of cells become damaged or die, or the cells lose their coupling with each other, the behavioral rhythm they drive will be lost or weakened while others remain. In this sense, aging can be considered similar to partial SCN lesions in young rats, reducing either the number of oscillators or the synchrony between them. Such lesions have been found to cause differential disruption of the three behavioral rhythms measured in the present experiments (16). Although there are no experiments characterizing coupling among individual SCN cells, there is evidence that there are cellular changes with aging. For instance, a loss of peptidergic neurons in the SCN of old rats has been reported (3, 14). Alternatively, in individual aging animals, the target brain areas for the three behavioral rhythms may be differentially sensitive to signals from the pacemaker.

The second point concerns changes in the activity pattern of neurons within the SCN itself. Examination of individual slice activity records shows that there are peaks in firing rate at times never seen in young rats. The curve of the average firing rate for the old rats (Fig. 3) shows that an underlying circadian rhythmicity remains in the SCN; peaks occur at the expected times on both *day 1* and *day 2*. The aberrant peaks and troughs are superimposed on this basic rhythm. However, the amplitude of the rhythm in old rats is significantly damped at the normal peak times.

The functional identity of SCN neurons based on firing pattern is presently unknown (4). The neuronal activity rhythms measured here are most likely outputs from the SCN clock and not necessarily integral constituents of the clock mechanism (17). Nevertheless, it may seem surprising that deterioration of circadian rhythmicity in this output from the pacemaker, or the coupling between its cellular elements, does not prevent entrainment in the overt behavioral rhythms. This may indicate that different outputs from the SCN maintain entrainment of different rhythms. This hypothesis assumes that the entraining signal (LD) is capable of generating rhythmic SCN output of some kind even if the population of neurons within the SCN does not sustain an autonomous rhythm. In any case, the present results demonstrate that there is no more correlation between the output of the SCN and the rhythmicity of the behavioral rhythms than there is among the behavioral rhythms themselves. Of course, behavioral rhythms were measured under entraining signals that were not present in vitro. We do not know whether an arrhythmic SCN in vitro would have been arrhythmic under the entraining conditions in vivo.

Previously, declines in rhythmicity in various behaviors could have been interpreted as declines in homeostatic abilities taking place downstream from the pacemaker. The present results demonstrate that aging affects endogenous rhythmicity far more upstream, indeed, within the SCN itself.

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Short Communications

Suprachiasmatic circadian pacemaker of rat shows two windows of sensitivity to neuropeptide Y in vitro

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Key words: Neuropeptide Y; Suprachiasmatic nuclei; Circadian rhythm; Pacemaker; Brain slice; Intergeniculate leaflet; Rat

The geniculohypothalamic tract carries visual information from the intergeniculate leaflet to the suprachiasmatic circadian pacemaker. NPY, found in this projection, has been shown to affect the phase of behavioral rhythms and influence photic entrainment. We now demonstrate that NPY, when briefly applied to the geniculate projection sites of rat SCN in vitro, induces permanent phase-shifts in the rhythm of neuronal electrical activity at two separate phases of the circadian cycle.

The suprachiasmatic nuclei (SCN) of the hypothalamus contain a circadian pacemaker, which acts to synchronize rhythmic processes of the organism with the daily rhythms in the environment[40,56]. The SCN clock has the ability to sustain near 24-h oscillations in isolation from external time signals[14], but it can be reset by environmental changes transmitted to the nuclei by neuronal and endocrine inputs. The SCN receive photic information via the retinohypothalamic tract (RHT) and the geniculohypothalamic tract (GHT). The RHT projects bilaterally from the retina to the ventrolateral regions of the SCN[21,42,48]. It carries primary visual information, about changes in environmental lighting, and it is essential for entrainment to light-dark cycles[19,26,27,50]. Ablation of the RHT results in the loss of photic entrainment even if other inputs to the SCN are intact[26].

RHT afferents, the axons of a specific subset of retinal ganglion cells, bifurcate at the SCN[39,40]. Some of these projections continue on to the lateral geniculate nucleus (LGN) of the thalamus. Among their postsynaptic sites are neurons of the intergeniculate leaflet (iGL). The rat iGL is a uniform lamina of neurons extending between the dorsal and ventral LGN[8,13,22,41,63]. In addition to bilateral retinal efferents[22], it receives serotonergic innervation from the dorsal raphe nuclei[10,11,41]. Neurons of the iGL in turn project back to the SCN, forming the GHT[6,8,62], as well as to the pineal gland[37], the source of the neuroendocrine signal of darkness, melatonin.

The retinorecipient cells of the iGL are immunoreactive for neuropeptide Y (NPY)[6,17,18], and in the rat they predominantly project to the ventrolateral region of the SCN via the GHT, to overlap with retinal terminals[7,8,38]. The GHT is not essential for entrainment to light–dark cycles but has been found to affect the size of the phase-shifts induced by light pulses as well as to influence photic entrainment. Lesions of the iGL in hamsters induce phase-shifts in activity rhythms[50] and alter the rate of reentrainment to varied lighting schedules[27,51]. Similarly, electrical stimulation of the GHT results in phase-shifts of hamster wheel running rhythms[36]. In addition, hamster responses to constant light are affected by GHT/iGL lesions. GHT lesioned hamsters are found to be less susceptible to splitting of activity rhythms[19], while a significant number of hamsters with split rhythms induced under constant light show fusion of activity rhythms following such lesions[19].

NPY is a 36 amino acid, C-terminally amidated peptide of the family of pancreatic polypeptides[64,65] that is thought to play a modulatory role in the hy-

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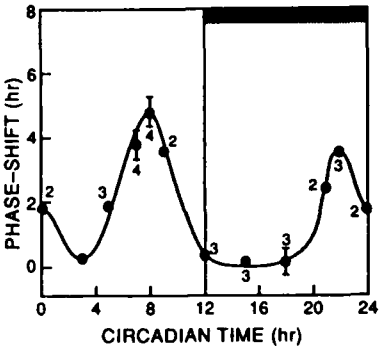

Fig. 1. Phase–response curve for NPY. The x-axis denotes the circadian time of NPY treatment (h) and the y-axis indicates the average magnitude and direction of the induced phase-shift (h). The magnitude of the shift in the time-of-peak in the electrical activity rhythm was determined in relation to time-of-peak in slices treated with microdrops of medium alone. Filled circles denote the mean ± S.E.M. phase-shift. The subscript number indicates the number of experiments performed at a particular circadian time. Points at CT 0 are replotted at CT 24. The vertical bar marks the time of 'lights-off' and the horizontal bar represents the subjective night in the colony.

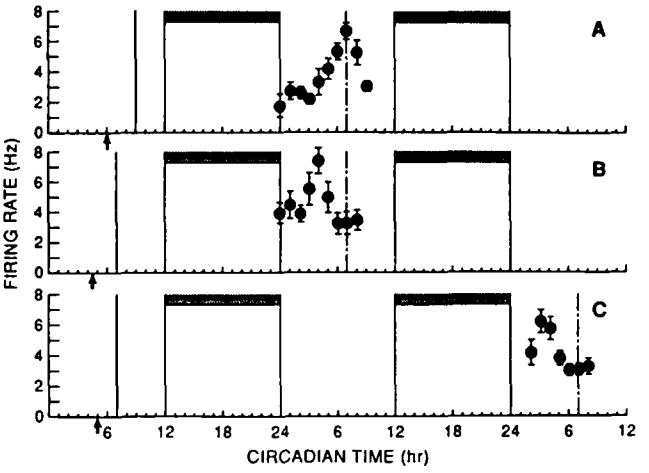

Fig. 2. NPY induces permanent phase-shifts in vitro. A: rhythm of endogenous neuronal activity recorded on day 2 after treatment with microdrops of EBSS on day 1 at CT 9. B: single SCN treated with a microdrop of 10^{-6} M NPY on day 1 at CT 7. The time-of-peak on day 2 occurred 3.25 h earlier than in control slices. C: recording on day 3, in a separate experiment, following treatment with NPY at CT 7 on day 1, resulted in a 3.5 h phase-advance. This is nearly 24 h later than the average time-of-peak seen on day 2 (3.75 ± 0.46 h). Filled circles denote the hourly means ± S.E.M. of the neuronal activity rhythm recorded on the second and third day. The vertical bar indicates the time of treatment. The interrupted line shows the time-of-peak observed in untreated slices and the horizontal stippled bar represents the time of the donor's night in the colony. The arrow points out the time of slice preparation.

Subsequently these were repeated with 10^{-6} M NPY/EBSS. There was no observable difference in the phase-shifts obtained. The average phase advance induced by NPY/dH_2O at CT 7 was 3.7 ± 0.52 h ($n = 3$), while NPY/EBSS resulted in a 4.0 h advance in the time-of-peak. Neither of the solutions induced a phase-shift in the electrical activity rhythm at CT 15 or CT 18. All following time points were tested using NPY/EBSS; the results from both procedures were found overlapping, and were grouped and averaged for analysis.

We also addressed the issue of whether NPY resets the pacemaker permanently. This was demonstrated by recording the rhythm of electrical activity on the second and third days in vitro, following treatment at CT 7 on day 1 (Fig. 2). The time-of-peak recorded on day 3 was at CT 3.5 ($\Phi_A = 3.5$ h), within the range of peak times recorded on day 2 in separate experiments (average $\Phi_A = 3.75 \pm 0.46$ h).

A dose–response curve was generated for NPY administered at CT 7 in microdrops, at 4 different concentrations, 10^{-5} M, 10^{-6} M, 10^{-8} M and 10^{-10} M (Fig. 3). Concentrations of 10^{-6} M (which were used in experiments addressing temporal sensitivities) elicited a maximal phase-shift of 3.75 ± 0.46 h, while the half maximal phase change was seen near 5×10^{-9} M. These results demonstrated that the phase-shifting effect of NPY on the circadian pacemaker is dose dependent.

We have shown that NPY can play a regulatory role in the mammalian circadian system by affecting the phase of the pacemaker directly. The dose–response relationship demonstrates that concentrations effective at inducing phase-shifts are in a physiological range. The actual effective dose is likely somewhat lower than that of the solution applied due to diffusion away from the site of microdrop application. Administration of microdrops of NPY at the GHT projection sites resulted in permanent advancing of the circadian clock during the mid to late subjective day and at the end of the subjective night.

There is notable similarity between the daytime sensitivity of the SCN to direct application of NPY and the daytime responses of animals to dark pulses[5] or to

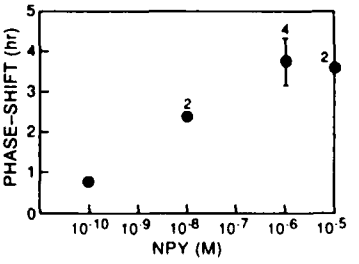

Fig. 3. NPY dose–response curve. Slices were treated with different concentrations of NPY ranging from 10^{-5} M to 10^{-10} M at CT 7. The x-axis denotes the NPY concentration (M) administered while the y-axis indicates the phase-shift response (h). The magnitude of the phase-shift in the time-of-peak was determined in relation to the peak time in vehicle microdrop-treated control slices. Filled circles represent the mean ± S.E.M. phase-shift. The subscript number indicates the number of experiments performed at a particular concentration.

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entrained 12L:12D cycle, by 7–3 h at dusk and 4–0 h at dawn. Interestingly, these NPY sensitive periods occur just before the rat SCN shows sensitivity to the pineal hormone, melatonin[32,33], the neuroendocrine signal of darkness. Because these sensitive periods are no doubt driven by the SCN clock, this observation raises the possibility that the substrates underlying these sensitivities are sequentially linked in the clock's mechanism.

This study has confirmed that NPY can play a regulatory role in the mammalian circadian system. Further, we have established the circadian timing of sensitivities to NPY, which anticipate the transitions in the entraining light–dark cycle. While NPY is not essential for generating circadian rhythmicity, there is increasing evidence suggesting that it is an integral element of the circadian system, providing behavior-coupled photic signals necessary for everyday entrainment to the environment.

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CIRCADIAN PATTERNS OF SUPRACHIASMATIC NEURONAL ACTIVITY AND SENSITIVITY

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The central role of the suprachiasmatic nuclei (SCN) in regulating circadian rhythms is well established. We have been using a hypothalamic brain slice preparation to study the temporal organization of rat SCN neuronal properties. This approach enables us to determine properties endogenous to the SCN.

All experiments were performed on tissue from our inbred colony of Long Evans rats, 6-8 months of age. Rats are reared in a 12L:12D schedule and fed *ad libitum*. Coronal slices of SCN-bearing hypothalamus (500 μM thick, containing less that the entire anterior-posterior extent of SCN) are placed in a perifusion-interface chamber where they are maintain at 37°C, bathed in Earles' Balanced Salt Solution (Sigma) supplemented to a final concentration of 24.6 mM glucose, 26.2 mM bicarbonate (pH 7.4) and gentamicin (0.0005%, Sigma) and exposed to a moist atmosphere of 95% O_2:5% CO_2. Slices have been studied for up to 53 hr. Spontaneous neuronal firing of single neurons is studied by extracellular recording techniques over the course of successive days *in vitro*. Averaged firing frequencies for individual neurons are used to study the circadian rhythms of electical activity and its modulation by cellular agents. Additionally, whole cell recording with patch electrodes is used to assess the electrical properties of individual SCN neurons.

SCN generate near 24-hr oscillations in ensemble neuronal firing rate for at least 3 days *in vitro*. The rhythm is sinusoidal, with peak activity appearing at CT 7.0 ± 0.1[1] (CT=circadian time, starting at CT 0 with "lights on" in the colony, and continuing for 24 hr). The time of peak activity of the neuronal population is stable across multiple cycles and predictable between animals, thus is used as a marker of phase. Individual neurons, however, exhibit diversity in their electrical properties, including firing rate, pattern and response to imposed current, at any one point in the circadian cycle. Further, there is temporal change in electrical properties over the course of the circadian cycle.[2] Both membrane potential and conductance show significant circadian variation, which may underlie circadian differences in responses to excitatory and inhibitory neurotransmitters.

In addition to changes in neuronal activity, the SCN *in vitro* undergo sequential changes in sensitivities to resetting agents over the 24-hr circadian cycle. During the subjective day the SCN clock progresses through periods of sensitivity to cAMP[1], serotonin[3], neuropeptide Y[4], and then to melatonin at dusk[5], while during the subjective night sensitivity to cGMP[6] is followed by another period of sensitivity to melatonin at dawn[7]. Intriguingly, agents that selectively stimulate cAMP or cGMP pathways have sensitive periods reminiscent of daytime and nighttime phase-shifting agent *in vivo*. These findings emphasize that the fundamental properties of a circadian clock survive in the SCN *in vitro*. Understanding the cellular mechanisms that generate these endogenous changes in the SCN time-keeping mechanism are basic to understanding the SCN's integrative and regulatory role for organismic rhythms such as sleep.

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GLUTAMATE INDUCES LIGHT-LIKE PHASE SHIFTS IN THE RAT SCN IN BRAIN SLICE.

J. M. Ding* and M. U. Gillette. Dept. of Cell & Structural Biology and Neuroscience Program, University of Illinois, Urbana, IL 61801.

The suprachiasmatic nucleus (SCN) receives direct visual input via the retino-hypothalamic tract (RHT). A number of behavioral, electrophysiological, and immunocytochemical studies suggest that glutamate (GLU) may play a role in photo-entrainment through the RHT pathway. We examined the effect of GLU on the phase of circadian rhythm of the electrical activity of the SCN in brain slice. Hypothalamic slices containing SCN from 7-10 wk male L-E rats in 12L:12D were treated focally with 1 µl GLU (10^{-7}-10^{-2} M, pH 7.4) for 10 min at different circadian times (CT). The phase of the circadian rhythm was determined by measuring the time-of-peak of the spontaneous discharge rate of the SCN in brain slice. The phase response curve (PRC) induced by GLU resembles the light-induced PRC with a maximum 4 hr ϕ_A at CT 19 and a 3 hr ϕ_D at CT 14-15. No phase shift was induced in the subjective day. This supports a role for GLU in photo-entrainment directly at the SCN. Furthermore, when GLU was applied at CT 17, the potential phase delay or advance transitional zone, multiple peaks of firing rate occurred, suggesting that within this particular time frame, the SCN might be intrinsically programmed to be able to shift to either direction.

(Supported by AFOSR grant 90-0205).

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1. Circadian
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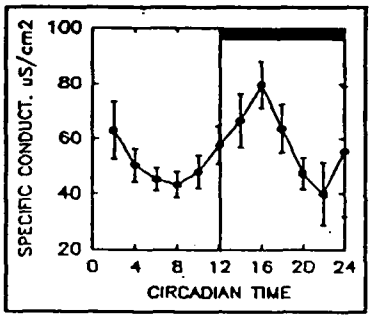
CIRCADIAN MODULATION OF MEMBRANE PROPERTIES OF SCN NEURONS IN RAT BRAIN SLICE

E.A. Gallman* and M.U. Gillette. Depts of Cell & Structural Biology and Physiology & Biophysics, Univ. of Illinois, Urbana, IL, 61801.

The suprachiasmatic nuclei (SCN) in rat are the neuroanatomic substrate for a pacemaker underlying circadian rhythmicity. The mechanisms responsible for circadian rhythmicity are unknown. Mean firing frequency of SCN neurons varies in a circadian pattern, with peak activity occurring near the middle of subjective day, both *in vivo* and in the *in vitro* hypothalamic brain slice. We employed whole cell recording in the rat brain slice to examine possible mechanisms for modulation of firing rate, as such mechanisms may reflect underlying organization of the circadian clock. We found statistically significant changes in membrane potential and specific membrane conductance which paralleled the changes in firing rate. Membrane potential was more negative at CT 14-18 than at CT 6-10. Specific membrane conductance was low at CT 6-10 and high at CT 14-18 (Figure). We are investigating the ionic conductances underlying these observations. AFOSR-90-0205 & PHS NS 22155.



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1. SUPRACHIASMATIC
2. PATCH CLAMP
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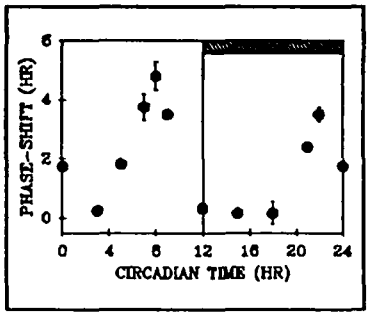
RAT SUPRACHIASMATIC CIRCADIAN PACEMAKER SHOWS TWO WINDOWS OF SENSITIVITY TO NPY IN VITRO.

M. Medanic* & M.U. Gillette. Depts of Physiology & Biophysics, and Cell & Structural Biology, Univ. of Illinois, Urbana, IL, 61801.

Neuropeptide Y (NPY), present in the geniculo-hypothalamic tract to the suprachiasmatic nuclei (SCN), is thought to influence photic as well as behavioral entrainment in the mammalian c[illegible]ian system. We have examined the role of NPY in rats by studying its effect on the electrical activity rhythm of SCN neurons *in vitro*. The SCN, isolated in hypothalamic brain slices from Long Evans rats (12L:12D), were briefly treated with a microdrop of NPY to the geniculate projection sites, at 11 time points across the circadian cycle. The effects of this treatment on the electrical activity rhythm were assessed extracellularly on day 2 and 3 *in vitro*. Phase shifts were determined by comparing the time-of-peak in NPY vs. vehicle treated slices. A microdrop of 10^{-6} M NPY was found to induce phase-shifts between CT 5-9 and CT 21-24, demonstrating two windows of SCN sensitivity which precede photic transitions in the entrained day-night cycle. (Supported by AFOSR Grant 90-0205).



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DO THE SUPRACHIASMATIC NUCLEI OSCILLATE IN OLD RATS AS THEY DO IN YOUNG ONES?

E. Satinoff*, H. Li, C. Liu, A. McArthur, M. Medanic, T. Tcheng and M. U. Gillette. Depts of Psychology, Physiology, Cell and Structural Biology and Neurosci. Prog. Univ. Illinois, Champaign, IL 61820

The basis of the decline in circadian rhythms with aging was addressed by comparing three behavioral rhythms in young and old rats with the rhythm of neuronal activity (NA) in the suprachiasmatic nuclei (SCN), the primary circadian pacemaker. In some old rats, rhythms of body temperature, drinking and activity retained significant 24-h periodicities in LD cycles; in others one or two of the rhythms became aperiodic. When these rats were 23-27.5 mo old, they were sacrificed, single unit firing rates in SCN brain slices were recorded for 30 h, and NA running averages were determined. NA from rats with prior aperiodic behavioral rhythms was erratic. NA from rats that still had 24-hr behavioral rhythmicity at the time of sacrifice was erratic in most cases but normally rhythmic in others. Thus, there was no more congruence between the behavioral rhythms and the brain slice rhythms than there was among the behavioral rhythms alone.

Because the brain slice releases each SCN into constant environmental conditions in vitro, we compared the data for SCN NA between groups of old and young rats. While both groups showed well defined peaks in ensemble neuronal firing rates on day 1 in vitro at the expected time, the mean rhythms of the old SCN were damped ($p < 0.001$) for the time bins that span time of peak activity, from CT 4-10. On day 2 in vitro, the old rats' mean NA was so damped ($p < 0.001$) that a peak was barely discernable. This change in amplitude reflects underlying changes in the behavior of individual NA such that there is apparent loss of coherence of the timing of appearance of fast units, producing multiple peaks, as well as a general decrease or disappearance of the most rapidly firing cells.

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